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(54) Title: USE OF NEUREGULINS AS MODULATORS OF CELLULAR COMMUNICATION

(57) Abstract

The present invention relates to methods of affecting cellular communication in a vertebrate. The communication is affected by the administration of a neuregulin to a vertebrate, where the neuregulin interacts with a first cell type which results in the production of a product (i.e., Product A). This product, in turn, affects the function of a second cell type. Methods are disclosed in which the affect in function of the second cell type, results in the production of a second product (i.e., Product B) which, in turn, can affect the function of the first cell type or a third cell type. Additional methods are included for treatment of disorders involving an altered or inadequate level of production of a product involved in cellular communication.

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USE OF NEUREGULINS AS MODULATORS OF CELLULAR COMMUNICATION

FIELD OF THE INVENTION

This invention relates to methods of affecting cellular communication.

BACKGROUND OF THE INVENTION

Vertebrate cells depend on externally produced factors for growth, differentiation and survival. These factors can be in the form of diffusible molecules that act at a distance from their site of synthesis. Alternatively these factors can be in the form of cell-surface-bound molecules that rely on cell-to-cell contact for their function. In many cases, different cell types may interact in a reciprocal manner in that both cell types produce factors that affect the other cell type. Vertebrates rely on these reciprocal interactions during embryogenesis and during the response to injury and disease.

Interdependence of cells and tissues plays important roles in the vertebrate nervous system. The nervous system is composed of neurons and neuroglial support cells. Peripheral nervous system axons are ensheathed by neuroglial cells (Schwann cells) and target organs which include skin, sensory receptors, muscle and other neurons. Additionally, peripheral axons interact with components of the central nervous system in the spinal cord. These include neurons and neuroglial cells such as astrocytes and oligodendrocytes .

It is well established that neurons and the tissues and cells with which they interact are dependent on each other for trophic support. This relationship is mediated by factors (proteins) produced by neurons that maintain the viability of target tissues (e.g. motor neuron derived factors that maintain muscle integrity) and neurotrophic factors produced by target (and other) tissues that maintain neuronal viability (e.g. muscle derived factors that maintain motor neuron viability). This interdependence plays an important role in embryonic development, maintenance of viability and response to injury in the nervous system and its targets.

The survival of various neuronal populations has been thought to be dependent only upon neurotrophic factors produced by targets of innervation. Recently it has been realized that neurotrophic factors are also derived from axonally associated cells

(periaxonal glia), soma associated (perisomatic) cells (e.g. glia and efferent synapses) and from autocrine sources. These proteins are taken up by neurons where they exert their effect at the cell body . Neurotrophic factors either maintain the viability of the neuron or induce specific effects such as axonal extension, sprouting and other responses to injury and disease. Examples include factors such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and related molecules as well as ciliary neurotrophic factor (CNTF), insulin like growth factor (IGF) and fibroblast growth factors (FGF's) that all have neurotrophic activity and are derived from neuronally associated tissues as diverse as muscle, Schwann cells and spinal cord astrocytes and other neurons (e.g., Nishi, *Science* (1994) **265**:1052).

The identification of pharmaceutical products or agents which induce the endogenous production of trophic factors would be beneficial treatment of diseases which involve trophic support.

SUMMARY OF THE INVENTION

In general, the present invention relates to methods of affecting cellular communication in a vertebrate. The communication is affected by the administration of a neuregulin to a vertebrate, where the neuregulin interacts with a first cell type which results in the production of a product or products (i.e., Product(s) A). This product, in turn, affects the function of a second cell type (see Figures 9 and 10).

Neuregulins are a family of protein factors encoded by one gene. A variety of messenger RNA splicing variants (and their resultant proteins) are derived from this gene and many of these products show binding and activation to erbB2 (neu) and closely related receptors erbB3 and erbB4. The invention provides methods for using all of the known products of the neuregulin gene, as well as, other not yet discovered splicing variants of the neuregulin gene.

Methods also are provided by the invention in which the effect in function of the second cell type, as described above, results in the production of a second product (i.e., Product B) which, in turn, can affect the function of the first cell type or a third cell type (see Figures 9 and 10).

Included in the invention as well, are methods for treatment when disorders involve an altered or inadequate level of production of a product involved in cellular communication.

Advantages of the present invention include the development of new therapeutic approaches to injury or disease based on the interdependence or communication of cells and the ability to influence or affect that communication with neuregulins. For example, a neuregulin factor that is produced by the second cell type can induce the first cell type to produce a product or products (Product(s) A) that are trophic for the second cell type. More specifically, cells and tissues that are associated with neurons may be induced to respond to a neuronally produced factor (neuregulin). This response would be in the form of the production of products (Product(s) A) that are trophic for neurons. The endogenous induction of more than one neurotrophic products by the neuregulin would be more effective than the therapeutic use of a single neurotrophic factor. Neurotrophic factors generally have restricted effects on specific neuronal subtypes (e.g. CNTF is trophic for motor neurons and NGF is trophic for sympathetic neurons as well as a subset of sensory neurons). Furthermore, the types of neurotrophic factors produced by a particular tissue are probably dependent on the target neuron type as well

as the type and stage of injury. As an example, CNTF, which is trophic for motor neurons, is released by Schwann cells in the early stages of recovery from nerve injury. This is replaced within a few days by Schwann cell and muscle derived BDNF, another motor neuron trophic factor (Curtis, et al., *Nature* (1993) **365**:253-255; and Funakoshi, et al, *J. Cell Biol.* (1993) **123**:455-465). In addition multiple neurotrophic factors function *in vivo* and may be synergistic in their effects. For example, it has been shown that multiple factors more efficiently arrest disease induced neuronal degeneration in animals than the use of a single factor (Mitsumoto et al., *Science* (1994) **265**:1107).

In the central nervous system, the neuregulin target, the first cell type, could be a neuron that in turn produces Product(s) A. Product A then affects other tissues (the second cell type) that produce neurotrophic products (Product(s) B) that affect the second cell type (the second cell type may be the source of the neuregulin), or perhaps a third cell type.

Thus, the use of the neuregulins, that are trophic for neuronally associated tissues in the manner described above would be therapeutically useful. Treatment would ensure the production of target specific combinations of products that are tailored to a particular disease state.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic diagram showing the method used to set up the SCG (superior cervical ganglion)/culture tube experiments; (1) tubes are filled with collagen +/- GGF; (2) SCG explants are placed in tubes; (3) tubes are cultured in humidified chambers; and (4) the extruded gels are fixed and stained as a "whole mount" (Anti-S100 for Schwann cells, and anti-tubulin β3 for axons.).

Figure 2 is a schematic diagram of the grid reticule inserted in the microscope ocular, which at a total magnification of 160X, allowed quantification of Schwann cell outgrowth and neurite outgrowth for the SCG/culture tube experiments.

Figure 3A shows the control data, that is, Schwann cell number as a function of distance from the SCG explant, for the SCG/culture tube experiments.

Figure 3B shows experimental data, of Schwann cell outgrowth for the SCG/culture tube experiments, at a dosage of 5 ng/ml rhGGF2.

Figure 3C shows experimental data, of Schwann cell outgrowth for the SCG/culture tube experiments, at a dosage of 50 ng/ml rhGGF2.

Figure 3D shows experimental data, of Schwann cell outgrowth for the SCG/culture tube experiments, at a dosage of 500 ng/ml rhGGF2.

Figure 4 shows the total number of Schwann cells as a function of days *in vitro* for the SCG/culture tube experiments.

Figure 5 shows experimental data, of neurite outgrowth, as a function of distance from the SCG explant, for the SCG/culture tube experiments performed at dosage levels of 5, 50 and 500 ng/ml rhGGF2.

Figure 6A shows a 2-dimensional dose-response matrix, used to examine the effects of rhGGF2 on neuronal survival and outgrowth.

Figure 6B illustrates the manner of counting, used in the afore-mentioned 2-dimensional dose-response experiment, by showing a representative sample well with fields of view.

Figure 7 shows experimental data of the effects of rhGGF2 on neuronal survival and outgrowth.

Figure 8A shows data on the effects of exogenous GGF on the number of myelinated axons at 28 days post-injury.

Figure 8B shows the above-referenced data in bar graph form.

Figure 9 represents a schematic illustration of the effect neuregulins can have on cellular communication.

Figure 10 represents a schematic illustration of specific effects of neuregulins on cellular communication within the nervous system.

Figure 11A is a listing of the coding strand DNA sequence and deduced amino aid sequence of the cDNA obtained from splicing pattern of GGF2BPP1 shown in Figure 12. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA);

Figure 11B is a listing of the coding strand DNA sequence and deduced amino acid sequence of the cDNA obtained from splicing pattern of GGF2BPP2. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA);

Figure 11C is a listing of the coding strand DNA sequence and deduced amino acid sequence of the cDNA obtained from splicing pattern of GGF2BPP3. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA).

Figure 12 is a diagram of representative splicing variants corresponding to bovine GGF gene products. The coding segments are represented by F, E, B, A, G, C, C/D, C/D', D, D', H, K and L. The location of the peptide sequences derived from purified protein are indicated by "o."

Figure 13 is a listing of the DNA sequences and predicted peptide sequences of the coding segments of GGF. Line 1 is a listing of the predicted amino acid sequences of bovine GGF, line 2 is a listing of the nucleotide sequences of bovine GGF, line 3 is a listing of the nucleotide sequences of human GGF (heregulin) (nucleotide base matches are indicated with a vertical line) and line 4 is a listing of the predicted amino acid sequences of human GGF/heregulin where it differs from the predicted bovine

sequence. Coding segments E, A' and K represent only the bovine sequences. Coding segment D' represents only the human (heregulin) sequence.

Figure 14 is the predicted GGF2 amino acid sequence and nucleotide sequence of BPP5. The upper line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

Figure 15 is the predicted amino acid sequence and nucleotide sequence of GGF2BPP2. The upper line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

Figure 16 is the predicted amino acid sequence and nucleotide sequence of GGF2BPP4. The upper line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

Figure 17 is a list of splicing variants derived from the sequences shown in Figure 13.

Figure 18 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL1.

Figure 19 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL2.

Figure 20 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL3.

Figure 21 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL4.

Figure 22 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL5.

Figure 23 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL6.

Figure 24 is the predicted amino acid sequence (middle) and nucleic sequence (top) of GGF2HBS5. The bottom (intermittent) sequence represents peptide sequences derived from GGF-II preparations.

Figure 25 is the sequences of GGFHBS5, GGFHB1 and GGFBPP5 polypeptides.

Figure 26 is the amino acid sequence of cDNA encoding mature hGGF2.

Figure 27 depicts a stretch of the putative bovine GGF-II gene sequence from the recombinant bovine genomic phage GGF2BG1. The figure is the coding strand of the DNA sequence and the deduced amino acid sequence in the third reading frame.

DETAILED DESCRIPTION OF THE INVENTION

It is intended that all references cited shall be incorporated herein by reference.

General

The invention pertains to methods of affecting cellular communication in vertebrates. The communication is affected by the administration of a neuregulin to a vertebrate where the neuregulin interacts with a first cell type which results in the production of a product. This product, in turn, affects the function of a second cell type. More specifically, the invention relates to the induction of endogenous tropic factors (products) by the administration of a neuregulin.

Methods also are provided by the invention in which the affect in function of the second cell type, described above, results in the production of a second product which, in turn, can affect the function of the first cell type, the second cell type or a third cell type.

Definition of Key Terms

The term administration as used herein refers to the act of delivering a substance, including but not limited to the following routes: parenteral, intravenous, subcutaneous, intramuscular, intraorbital, ophthalmic, intraventricular, intracranial, intracapsular, intraspinal, intracisternal, intraperitoneal, topical, intranasal, aerosol, scarification, orally, buccal, rectal or vaginal. Administration as used herein refers to a pharmaceutical preparation of a substance and the delivery of that preparation to a recipient.

The term affecting as used herein refers to the induction of a quantitative change in the response of a target cell, as a result of an interaction with a Product.

The term Alzheimer's Disease as used herein refers to a progressive central neurodegeneration involving loss of cortical and other neurons, and associated with neurofibrillary tangles and ß-amyloid deposits.

The term amyotrophic lateral sclerosis (ALS) as used herein refers to a motor neuron disease characterized by a progressive degeneration of the neurons that give rise to the corticospinal tract that results in a deficit in upper and lower motor neurons.

The term astrocyte as used herein refers to a neuroglial cell of ectodermal origin and its progenitor cells. This cell has a round nucleus and a "star shaped" body and many long processes that end as vascular foot plates on the small blood vessels of the CNS and is associated with other structures. A more complete definition of the astrocyte and its progenitors can be found in the following materials: Reynolds and Weiss, *Science* (1992) **255**:1707-1710; Reynolds, Tetzlaff, and Weiss, *J. Neurosci* (1992) **12**:4565-4574; and Kandel, et al., *Principles of Neuroscience*, third ed. (1991), Appleton & Lange, Norwalk, CT.

The term cellular communication as used herein refers to the synthesis of a substance in one cell type and the interaction of that substance with a second cell type. This process includes, but is not limited to, secretion of the substance from a cell. The substance elicits a change in the second cell type or with the first cell type. Communication can occur reciprocally or non-reciprocally with one or more cell types.

The term differentiation as used herein refers to a morphological and/or chemical change that results in the generation of a different cell type or state of specialization. The differentiation of cells as used herein refers to the induction of a cellular developmental program which specifies one or more components of a cell type. The therapeutic usefulness of differentiation can be seen, in increases in quantity of any component of a cell type in diseased tissue by at least 10% or more, more preferably by 50% or more, and most preferably by more than 100% relative to the equivalent tissue in a similarly treated control animal.

The term disorder as used herein refers to a disturbance of function and/or structure of a living organism, resulting from an external source, a genetic predisposition, a physical or chemical trauma, or a combination of the above, including but not limited to any mammalian disease.

The term first cell type as used herein refers to the cell type that interacts with a neuregulin. The first cell type includes but is not limited to one or more of the following: neuron, glial cell, Schwann cell, astrocyte, oligodendrocyte, myoblast, muscle cell, satellite cell, skin cell, sensory organ cell, inflammatory cell such as macrophage, neutrophil, T-cell, eosinophil, mast cell, basophil and stromal cell such as fibroblasts or endothelial cells. Bloom and Fawcett, *A Textbook of Histology*, tenth ed. (1975), W. B. Saunders Company, Philadelphia, PA.

The term function as used herein refers to any activity or response of a cell. These include but are not limited to proliferation, differentiation, growth, survival, changes in the pattern of gene expression and secretion, and metabolic changes.

The term glial cell as used herein refers to connective and support tissues of the nervous system and includes ectodermally derived astrocytes, oligodendroglia, Schwann cells and mesodermally derived microglia and their progenitors. A more complete definition of glial cells and their progenitors can be found in the following materials: Anderson, *FASEB J.* (1994) 8:707-713; Reynolds and Weiss, *Science* (1992) 255:1707-1710; Reynolds, Tetzlaff, and Weiss, *J. Neurosci* (1992) 12:4565-4574; and Kandel, et al., *Principles of Neuroscience*, third ed. (1991), Appleton & Lange, Norwalk, CT.

The term interacts as used herein refers to a contact with a target (cell), including but not limited to binding of a product to a cell receptor.

The term mammal as used herein describes a member of the Class Mammalia (Subphylum Vertebrata).

The term matrix molecule as used herein refers to a chemical component of the insoluble meshwork of extracellular proteins that mediate adhesive interactions between cells and modulate the functions of cells.

The term mitosis as used herein refers to the division of a cell where each daughter nucleus receives identical complements of the numbers of chromosomes characteristic of the somatic cells of the species. Mitosis as used herein refers to any cell division which results in the production of new cells in the patient. More specifically, a useful therapeutic is defined *in vitro* as an increase in mitotic index relative to untreated cells of 50%, more preferably 100%, and most preferably 300%, when the cells are exposed to labeling agent for a time equivalent to two doubling times. The mitotic index is the fraction of cells in the culture which have labeled nuclei when grown in the presence of a tracer which only incorporates during S phase (i.e., BrdU) and the doubling time is defined as the average time required for the number of cells in the culture to increase by a factor of two.

For example, one effect on mitosis *in vivo* is defined as an increase in satellite cell activation as measured by the appearance of labeled satellite cells in the muscle tissue of a mammal exposed to a tracer which only incorporates during S phase (i.e.,

BrdU). A useful therapeutic is defined *in vivo* as a compound which increases satellite cell activation relative to a control mammal by at least 10%, more preferably by at least 50%, and most preferably by more than 200% when the mammal is exposed to labeling agent for a period of greater than 15 minutes and tissues are assayed between 10 hours and 24 hours after administration of the mitogen at the therapeutic dose.

The term muscle cell as used herein refers to a cellular component of skeletal, smooth or cardiac muscle, including but not limited to myofibrils, satellite cells, and myoepithelial cells and their progenitors. A more complete definition of muscle cells can be found in, Wheater, et al., *Functional Histology* (1987), Churchill Livingstone, New York, NY; and *Myology*, ed. by Engel and Franzini-Armstrong, second ed. (1994) McGraw Hill, New York, NY.

The term neuregulin as used herein refers to the glial growth factors, the heregulins, neu differentiation factor, acetylcholine receptor inducing activity, and erbB2, 3 and 4 binding proteins. A more complete definition of neuregulins can be found in the specification herein and in the following materials: U.S. Patent No. 5,237,056; U.S. Patent Application SN 08/249,322; WO 92/20798; EPO 0 505 148 A1; Marchionni, et al., *Nature* **362**:313, 1993; Benveniste, et al., *PNAS* **82**:3930-3934, 1985; Kimura, et al., *Nature* (1990) **348**:257-260; Davis and Stroobant, *J. Cell. Biol.* (1990) **110**:1353-1360; Wen, et al., *Cell* (1992) **69**:559; Yarden and Ullrich, *Ann. Rev. Biochem.* (1988) 57:443, ; Holmes, et al., *Science* **256**:1205, 1992; Dobashi, et al., *Proc. Nat'l. Acad. Sci.* **88**:8582, 1991; Lupu, et al., *Proc. Nat'l. Acad. Sci.* (1992) **89**:2287; Peles and Yarden, *BioEssays* (1993) **15**:815, Mudge, *Current Biology* (1993) **3**:361, all hereby incorporated by reference.

The term neuregulin producing cell as used herein refers to a cell that produces a neuregulin. The term refers to all producer cells including cells that produce recombinant neuregulins.

The term neurological disorder as described herein refers to a disorder of the nervous system.

The term nervous system cell as used herein includes nervous system support cells and neurons.

The term neuron as used herein refers to a complete nerve cell, including the cell body and all of its processes, and its progenitors. A more complete definition of neuron

and its progenitors can be found in the following materials: Reynolds and Weiss, *Science* (1992) 255:1707-1710; Reynolds, Tetzlaff, and Weiss, *J. Neurosci* (1992) 12:4565-4574; Ray, Peterson, Schinstine, and Gage, *PNAS* (1993) 90:3602-3606; and Kandel, et al., *Principles of Neuroscience*, third ed. (1991), Appleton & Lange, Norwalk, CT.

The term neurotrophic agent as used herein refers to a substance that elicits a trophic effect in one or more neuronal subtypes. These effects include but are not limited to survival, sprouting and differentiation.

The term oligodendrocyte as used herein refers to the neuroglial cells, of ectodermal origin, with small oval nuclei and fine cytoplasmic processes that are responsible for the formation of myelin in the CNS. The progenitors of oligodendrocytes are also included. A more complete definition of oligodendrocytes and their progenitors can be found in Kandel, et al., *Principles of Neuroscience*, third ed. (1991), Appleton & Lange, Norwalk, CT.

The term Parkinson's Disease as used herein refers to a progressive central neurodegeneration involving dopaminergic neurons.

The term peripheral neuropathy as used herein refers to functional disturbances and/or pathological changes in the peripheral nervous system.

The term Product as used herein refers to any substance as defined herein as Product A or Product B.

The term Product A as used herein refers to the substances whose synthesis and release are induced in the first cell type by neuregulin. Such substances include but are not limited to one or more of the following: nerve growth factor (NGF), neurotrophins, brain-derived neurotrophic factor, ciliary neurotrophic factor, leukemia inhibiting factor, interleukin 6, platelet derived growth factor, fibroblast growth factors, transforming growth factor ß, epidermal growth factor, transforming growth factor a, neuregulins, insulin like growth factor, matrix molecules, adhesion molecules, growth factor receptors, low affinity NGF receptor, proteases, protease inhibitors, and antioxidants.

The term Product B as used herein refers to the substances whose synthesis and release are induced in the second cell type by Product A. Such substances include but

are not limited to one or more of the following: nerve growth factor (NGF), neurotrophins, brain-derived neurotrophic factor, ciliary neurotrophic factor, leukemia inhibiting factor, interleukin 6, platelet derived growth factor, fibroblast growth factors, transforming growth factor ß, epidermal growth factor, transforming growth factor a, neuregulins, glial derived neurotrophic factor, insulin like growth factor, matrix molecules, adhesion molecules, growth factor receptors, low affinity NGF receptor (p75), proteases, protease inhibitors and antioxidants.

The term production as used herein refers to induced or constitutive synthesis and/or release of a Product from a cell.

The term protease as used herein refers to an enzyme that hydrolyses peptide bonds in a protein molecule.

The term protease inhibitor as used herein refers to a molecule that inhibits the activity and/or function of a protease.

The term Schwann cell as used herein refers to the neuroglial cell composing the neurolemma of peripheral nerve fibers and its progenitors. A more complete definition of Schwann cells and their progenitors can be found in the following materials: Anderson, *FASEB J.* (1994) 8:707-713; Kandel, et al., *Principles of Neuroscience*, third ed. (1991), Appleton & Lange, Norwalk, CT.

The term second cell type as used herein refers to the cell type that interacts with and responds to Product A. The second cell type includes but is not limited to one or more of the following: neuron, glial cell, Schwann cell, astrocyte, oligodendrocyte, myoblast, muscle cell, satellite cell, skin cell, sensory organ cell, inflammatory cell such as macrophage, neutrophil, T-cell, eosinophil, mast cell, basophil and stromal cell such as fibroblasts or endothelial cells. A more complete definition may be found in Bloom and Fawcett, *A Textbook of Histology*, tenth ed. (1975), W. B. Saunders Company, Philadelphia, PA.

The term sensory organ cell as used herein refers to a primary sensory cell contained within a sensory organ and its progenitors and includes but is not limited to one or more of the following: taste cells, olfactory epithelial cell, rod and cone photoreceptors, Meisner corpuscle, Ruffini corpuscle, Merckel receptor, Pacinian corpuscle, muscle spindle cell, cochleovestibular hair cells and joint mechanoreceptor cells. A more complete definition of sensory organ cells and their progenitors can be

found in, Wheater, et al., *Functional Histology* (1987), Churchill Livingstone, New York, NY; Mahanthappa and Schwarting, *Neuron* (1993) **10**:293-305; Forge, Li, Corwin and Nevill, *Science* (1993) **259**:1616-1622; Tsue, Watling, Weisleder, Coltrera and Rubel, *J. Neurosci* (1994) **14**:140-152.

The term skin cell as used herein refers to the cellular components of the skin and includes fibroblasts, keratinocytes, epidermal cells, hair follicle cells, melanocytes, myoepithelial sweat gland cells, and sebaceous gland cells and their progenitors. A more complete definition of skin cells and their progenitors can be found in, Wheater, et al., *Functional Histology* (1987), Churchill Livingstone, New York, NY.

The term spinal muscular atrophy as used herein refers to a progressive disease of upper and lower motor neurons, usually present in childhood.

The term survival as used herein refers to any process where a cell avoids death. The term survival as used herein also refers to the prevention of cell loss as evidenced by necrosis or apoptosis or the prevention of other mechanisms of cell loss. Survival as used herein indicates a decrease in the rate of cell death of at least 10%, more preferably by at least 50%, and most preferably by the least 300% relative to an untreated control. The rate of survival may be measured by counting cells stainable with a dye specific for dead cells (such as propidium iodide) in culture when the cells are 8 days post-differentiation (i.e., 8 days after the media is changed from 20% to 0.5% serum).

The term therapeutically effective amount as used herein refers to that amount which will produce a desirable result upon administration and which will vary depending upon a number of issues, including the dosage to be administered, and the route of administration.

The term third cell type as used herein refers to a cell type that interacts with and responds to Product B. The third cell type may be identical to the first cell type. The third cell type includes but is not limited to one or more of the following: neuron, glial cell, Schwann cell, astrocyte, oligodendrocyte, myoblast, muscle cell, satellite cell, skin cell, sensory organ cell, inflammatory cell such as macrophage, neutrophil, T-cell, eosinophil, mast cell, basophil and stromal cell such as fibroblasts or endothelial cells. A more complete definition may be found in Bloom and Fawcett, *A Textbook of Histology*, tenth ed. (1975), W. B. Saunders Company, Philadelphia, PA.

The term treating as used herein may refer to a procedure (e.g. medical procedure) designed to exert a beneficial effect on a disorder. Treating as used herein means any administration of a substance described herein for the purpose of increasing cellular communication of products. Most preferably, the treating is for the purpose of reducing or diminishing the symptoms or progression of a disease or disorder of cells. Treating as used herein also means the administration of a substance to increase or alter the cells in healthy individuals. The treating may be brought about by the contacting of the cells which are sensitive or responsive to the neuregulins described herein with an effective amount of the neuregulin.

The term trophic as used herein refers to an effect of a substance on a cell, including but not limited to proliferation, growth, sprouting, differentiation or survival.

The term vertebrate as used herein refers to an animal that is a member of the Subphylum Vertebrata (Phylum Chordata).

Neuregulins

A novel aspect of the present invention relates to the ability of neuregulins to affect cellular communication between different and similar cell types. Neuregulins are the products of a gene which produce a number of variably-sized, differentially-spliced RNA transcripts that give rise to a series of proteins. These proteins are of different lengths and contain some common peptide sequences and some unique peptide sequences. The conclusion that these factors are encoded by a single gene is supported by the differentially-spliced RNA sequences which are recoverable from bovine posterior pituitary, human spinal chord and human breast cancer cells (MDA-MB-231). Further support for this conclusion derives from the size range of proteins which act as ligands for the p185^{erbB2} receptor (see below).

Further evidence to support the fact that the genes encoding GGF/p185^{erbB2} binding proteins are homologous comes from nucleotide sequence comparison. Holmes et al., (*Science* (1992) **256**:1205-1210) demonstrate the purification of a 45-kilodalton human protein (Heregulin-α) which specifically interacts with the receptor protein p185^{erbB2}. Peles et al., (*Cell* (1992) **69**:559) describe a complementary DNA isolated from rat cells encoding a protein call "neu differentiation factor" (NDF). The translation product of the NDF cDNA has p185^{erbB} binding activity. Several other groups have reported the purification of proteins of various molecular weights with p185^{erbB2} binding activity. These groups include the following: Lupu et al., (1992) *Proc. Nat'l. Acad. Sci. USA* **89**:2287; Yarden and Peles, (1991) *Biochemistry* **30**:3543; Lupu et al., (1990) *Science* **249**:1552; Dobashi et al., (1991) *Biochem. Biophys. Res. Comm.* **179**:1536; and Huang et al., (1992) *J. Biol. Chem.* **257**:11508-11512.

We have found that p185^{erbB2} and related receptor binding proteins (i.e., p185^{erbB3} and p185^{erbB4}) affect cellular communication. This effect results in the production of a product from a first cell type, where the product, in turn affects the function of a second cell type. The affect in a function of the second cell type and can result in the production of other products which also can affect functions of other cell types. For example, neuregulins can interact with Schwann cells, which as a result of this interaction produce neurotrophic agents. These agents, in turn, interact with neurons to promote their neuronal regeneration. Alternatively, in the central nervous system, a first cell type, being a neuron, could produce a neuregulin, which in turn, affects a second cell type which is a neuron also.

These neuregulins may be identified using the protocols described herein (Examples 1 and 2) and in Holmes et al., *Science* (1992) **256**: 1205; Peles et al., *Cell* (1992) **69**:205; Wen et al., *Cell* (1992) **69**:559; Lupu et al., *Proc. Nat'l. Acad. Sci. USA* (1992) **89**:2287; Yarden and Peles, *Biochemistry* (1991) **30**:3543; Lupu et al., *Science* (1990) 249:1552; Dobashi et al., *Biochem. Biophys. Res. Comm.* (1991) **179**:1536; Huang et al., *J. Biol. Chem.* (1992) **257**:11508-11512; Marchionni et al., *Nature* (1993) **362**:313; and in U.S. Patent Application Serial No. 07/931,041, filed August 17, 1992, all of which are incorporated herein by reference.

Specifically, the invention provides for use of polypeptides of a specified formula, and DNA sequences encoding those polypeptides. The polypeptides have the formula

WYBAZCX

wherein WYBAZCX is composed of the amino acid sequences shown in Figure 13; wherein W comprises the polypeptide segment F, or is absent; wherein Y comprises the polypeptide segment E, or is absent; wherein Z comprises the polypeptide segment G or is absent; and wherein X comprises the polypeptide segments C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, or C/D C/D' D' HKL; provided that, either

a) at least one of F, Y, B, A, Z, C, or X is of bovine origin; or

b) Y comprises the polypeptide segment E; or

c) X comprises the polypeptide segments C/D HKL, C/D D, C/D' HKL, C/D C/D' HKL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, C/D C/D' D' HKL, C/D'H, C/D C/D'H, or C/D C/D' HL.

In addition, the invention includes the use of the DNA sequence comprising coding segments $^{5'}$FBA$^{3'}$ as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 13;

the DNA sequence comprising the coding segments $^{5'}$FBA'$^{3'}$ as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 13;

the DNA sequence comprising the coding segments $^{5'}$FEBA$^{3'}$ as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 13;

the DNA sequence comprising the coding segments $^{5'}$FEBA'$^{3'}$ as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 13;

the DNA sequence comprising the polypeptide coding segments of the GGF2HBS5 cDNA clone (ATCC Deposit No. 75298, deposited September 2, 1992), also known as GGF-II.

The invention further includes the use of peptides of the formula FBA, FEBA, FBA' FEBA' and DNA sequences encoding these peptides wherein the polypeptide segments correspond to amino acid sequences shown in Figure 13. The purified GGF-II polypeptide is also included as part of the invention.

Also included in this invention is the mature GGF peptide and the DNA encoding said peptide, exclusive of the N-terminal signal sequence, which is also useful for treatment of conditions involving abnormalities in cellular communication.

Furthermore, the invention includes a method of cellular communication by the application to a vertebrate of a

- 30 kD polypeptide factor isolated from the MDA - MB 231 human breast cell line; or

- 35 kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line to the glial cell; or

-75 kD polypeptide factor isolated from the SKBR-3 human breast cell line; or

-44 kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line, or

-25kD polypeptide factor isolated from activated mouse peritoneal macrophages; or

-45 kD polypeptide factor isolated from the MDA - MB 231 human breast cell; or

-7 to 14 kD polypeptide factor isolated from the ATL-2 human T-cell line to the glial cell; or

-25 kD polypeptide factor isolated from the bovine kidney cell; or

-42 kD polypeptide factor (ARIA) isolated from brains.

The invention further includes a method for the use of the EGFL1, EGFL2, EGFL3, EGFL4, EGFL5 and EGFL6 polypeptides, Figure 18 to Figure 23 respectively, and for the methods of affecting cellular communication *in vivo* and *in vitro*.

Also included in the invention is the administration of the GGF-II polypeptide whose sequence is shown in Figure 24, for affecting cellular communication.

An additional aspect of the invention includes the use of the above-referenced peptides for the purpose of stimulating Schwann cells to produce growth factors which may, in turn, be harvested for scientific or therapeutic use.

Thus, the invention further embraces a polypeptide factor capable of affecting cellular communication and including an amino acid sequence encoded by:

(a) a DNA sequence shown in Figure 11;

(b) a DNA sequence shown in Figure 27;

(c) the DNA sequence represented by nucleotides 281-557 of the sequences shown in Figure 11; or

(d) a DNA sequence hybridizable to any one of the DNA sequences according to (a), (b) or (c).

The invention further includes sequences which have greater than 60%, preferably 80%, sequence identity of homology to the sequences indicated above.

While the present invention is not limited to a particular set of hybridization conditions, the following protocol gives general guidance which may, if desired, be followed:

DNA probes may be labeled to high specific activity (approximately 10^8 to 10^9 ^{32} Pdmp/μg) by nick-translation or by PCR reactions according to Schowalter and Sommer (*Anal. Biochem.* (1989) 177:90-94) and purified by desalting on G-150 Sephadex columns. Probes may be denatured (10 minutes in boiling water followed by immersion into ice water), then added to hybridization solutions of 80% buffer B (2g polyvinylpyrolidine, 2g Ficoll-400, 2g bovine serum albumin, 50ml 1M Tris HCl (pH 7.5), 58g NaCl, 1g sodium pyrophosphate, 10g sodium dodecyl sulfate, 950 ml H_2O) containing 10% dextran sulfate at 10^6 dpm ^{32}P per ml and incubated overnight (approximately 16 hours) at 60° C. The filters may then be washed at 60° C first in

buffer B for 15 minutes followed by three 20-minute washes in 2X SSC, 0.1% SDS then one for 20 minutes in 1XSSC, 0.1% SDS.

In other respects, the invention provides:

(a) a basic polypeptide factor which has, if obtained from bovine pituitary material, an observed molecular weight, whether in reducing conditions or not, of from about 30kD to about 36kD on SDS-polyacrylamide gel electrophoresis using the following molecular weight standards:

Lysozyme (hen egg white)	14,400
Soybean trypsin inhibitor	21,500
Carbonic anhydrase (bovine)	31,000
Ovalbumin (hen egg white)	45,000
Bovine serum albumin	66,200
Phosphorylase B (rabbit muscle)	97,400;

which factor has glial cell mitogenic activity including stimulating the division of rat Schwann cells in the presence of fetal calf plasma, and when isolated using reversed-phase HPLC retains at least 50% of said activity after 10 weeks incubation in 0.1 % trifluoroacetic acid at 4° C; and

(b) a basic polypeptide factor which has, if obtained from bovine pituitary material, an observed molecular weight, under non-reducing conditions, of from about 55 kD to about 63 kD on SDS-polyacrylamide gel electrophoresis using the following molecular weight standards:

Lysozyme (hen egg white)	14,400
Soybean trypsin inhibitor	21,500
Carbonic anhydrase (bovine)	31,000
Ovalbumin (hen egg white)	45,000
Bovine serum albumin	66,200
Phosphorylase B (rabbit muscle)	97,400;

which factor the human equivalent of which is encoded by DNA clone GGF2HBS5 described herein and is capable of affecting cellular communication.

For convenience of description only, the lower molecular weight and higher molecular weight factors of this invention are referred to hereafter as "GGF-I" and "GGF-II", respectively. The "GGF2" designation is used for all clones isolated with peptide sequence data derived from GGF-II protein (i.e., GGF2HBS5, GGF2BPP3).

It will be appreciated that the molecular weight range limits quoted are not exact, but are subject to slight variations depending upon the source of the particular polypeptide factor. A variation of, say, about 10% would not, for example, be impossible for material from another source.

Another important aspect of the invention is a DNA sequence encoding a polypeptide capable of affecting cellular communication and comprising:

(a) a DNA sequence shown Figure 11;

(b) a DNA sequence shown in Figure 27;

(c) the DNA sequence represented by nucleotides 281-557 of the sequence shown in Figure 11; or

(d) a DNA sequence hybridizable to any one of the DNA sequences according to (a), (b) or (c).

Thus other important aspects of the invention are:

(a) A series of human and bovine polypeptide factors capable of affecting cellular communication. These peptide sequences are shown in Figures 13, 14, 15 and 16, respectively.

(b) A series of polypeptide factors capable of affecting cellular communication and purified and characterized according to the procedures outlined by Lupu et al., *Science* (1990) **249**:1552; Lupu et al., *Proc. Nat'l. Acad. Sci USA* (1992) **89**: 2287; Holmes et al., *Science* (1992) **256**:1205; Peles et al., *Cell* (1992) **69**:205; Yarden and Peles, *Biochemistry* (1991) **30**:3543; Dobashi et al., *Proc. Nat'l. Acad. Sci.* (1991) **88**: 8582; Davis et al., *Biochem. Biophys. Res. Commun.* (1991) **179**:1536 ; Beaumont et al., Patent Application PCT/US91/03443 (1990); Greene et al., Patent Application PCT/US91/02331 (1990); Usdin and Fischbach, *J. Cell. Biol.* (1986) **103**:493-507; Falls et al., *Cold Spring Harbor Symp. Quant. Biol.* (1990) **55**:397-406; Harris et al., *Proc. Nat'l. Acad. Sci. USA* (1991) **88**:7664-7668; and Falls et al., *Cell* (1993) **72**:801-815.

(c) A polypeptide factor (GGFBPP5) capable of affecting cellular communication. The amino acid sequence is shown in Figure 14, and is encoded by the bovine DNA sequence shown in Figure 14.

The novel human peptide sequences described above and presented in Figures 13, 14, 15, and 16, respectively, represent a series of splicing variants which can be isolated as full length complementary DNAs (cDNAs) from natural sources (cDNA libraries prepared from the appropriate tissues) or can be assembled as DNA constructs with individual exons (e.g., derived as separate exons) by someone skilled in the art.

Other compounds in particular, peptides, which bind specifically to the $p185^{erbB2}$ receptor and related receptors can also be used according to the invention as affections of cellular communication. A candidate compound can be routinely screened for $p185^{erbB2}$ binding, and, if it binds, it can then be screened for affecting cellular communication using the methods described herein.

The invention includes any modifications or equivalents of the above polypeptide factors which do not exhibit a significantly reduced activity. For example, modifications in which amino acid content or sequence is altered without substantially adversely affecting activity are included. By way of illustration, in EP-A 109748, mutations of native proteins are disclosed in which the possibility of unwanted disulfide bonding is avoided by replacing any cysteine in the native sequence which is not necessary for biological activity with a neutral amino acid. The statements of effect and use contained herein are therefore to be construed accordingly, with such uses and effects employing modified or equivalent factors being part of the invention.

The new sequences of the invention open up the benefits of recombinant technology. The invention thus also includes the following aspects:

(a) DNA constructs comprising DNA sequences as defined above in operable reading frame position within vectors (positioned relative to control sequences so as to permit expression of the sequences) in chosen host cells after transformation thereof by the constructs (preferably the control sequence includes regulatable promoters, e.g. Trp). It will be appreciated that the selection of a promoter and regulatory sequences (if any) are matters of choice for those of skill in the art:

(b) host cells modified by incorporating constructs as defined in (a) immediately above so that said DNA sequences may be expressed in said host cells - the choice of host is not critical, and chosen cells may be prokaryotic or eukaryotic and may be genetically modified to incorporate said constructs by methods known in the art; and,

(c) a process for the preparation of factors as defined above comprising cultivating the modified host cells under conditions permitting expression of the DNA sequences. These conditions can be readily determined, for any particular embodiment, by those of skill in the art of recombinant DNA technology. Glial cell mitogens prepared by this means are included in the present invention.

None of the factors described in the art has the combination of characteristics possessed by the present new polypeptide factors.

The invention also includes a neuregulin as defined above, by extracting vertebrate brain material to obtain protein, subjecting the resulting extract to chromatographic purification by hydroxyapatite HPLC and then subjecting these fractions to SDS-polyacrylamide gel electrophoresis. The fraction which as an observed molecular weight of about 30kD to 36kD and/or the fraction which has an observed molecular weight of about 55kD to 63kD is collected. In either case, the fraction is subjected to SDS-polyacrylamide gel electrophoresis using the following molecular weight standards:

Lysozyme (hen egg white)	14,400
Soybean trypsin inhibitor	21,500
Carbonic anhydrase (bovine)	31,000
Ovalbumin (hen egg white)	45,000
Bovine serum albumin	66,200
Phosphorylase B (rabbit muscle)	97,400

In the case of the smaller molecular weight fraction, the SDS-polyacrylamide gel is run in non-reducing conditions or in reducing conditions and in the case of the larger molecular weight fraction, the gel is run under non-reducing conditions. The fractions are then tested for activity stimulating the division of rat Schwann cells against a background of fetal calf plasma.

Preferably, the above process starts by isolating a relevant fraction obtained by carboxymethyl cellulose chromatography, e.g. from bovine pituitary material. It is also preferred that hydroxyapatite HPLC, cation exchange chromatography, gel filtration, and/or reversed-phase HPLC be employed prior to the SDS-Polyacrylamide gel electrophoresis. At each stage in the process, activity may be determined using Schwann cell incorporation of radioactive iododeoxyuridine as a measure in an assay generally as described by Brockes in *Meth. Enz.* (1987) **147**:217-225, but modified by substituting 10% FCP for 10% FCS. As already noted, such as assay is an aspect of the invention in its own substance for CNS or PNS cell, e.g. Schwann cell, mitogenic effects.

Compounds may be assayed for their usefulness *in vitro* using the methods provided in the description and examples below. Following the *in vitro* demonstration of the effect of neuregulins on cellular communication between various cell types, the *in vivo* therapeutic benefit of the effect can be accomplished by the administration of neuregulins, neuregulin producing cells or DNA encoding neuregulins to a vertebrate

requiring therapy. In a specific example, *in vivo* testing can be demonstrated as described in Example 3.

The invention includes the use of the above named family of proteins (i.e. neuregulins) as extracted from natural sources (tissues or cell lines) or as prepared by recombinant means.

Other compounds in particular, peptides, which bind specifically to the p185erbB2 and related receptor binding proteins (i.e., p185erbB3 and p185erbB4) can also be used according to the invention as affectors of cellular communication. A candidate compound can be routinely screened for p185erbB2, p185erbB3 and p185erbB4 binding, and if it binds, can then be screened for affecting cellular communication using the methods described herein.

The invention includes use of any modifications or equivalents of the above polypeptide factors which do not exhibit a significantly reduced activity related to affecting cellular communication. For example, modifications in which amino acid content or sequence is altered without substantially adversely affecting activity are included. The statements of effect and use contained herein are therefore to be construed accordingly, with such uses and effects employing modified or equivalent factors being part of the invention.

The human peptide sequences described above represent a series of splicing variants which can be isolated as full length complementary DNAs (cDNAS) from natural sources (cDNA libraries prepared from the appropriate tissues) or can be assembled as DNA constructs with individual exons (e.g., derived as separate exons) by someone skilled in the art.

The invention includes methods for the use of any protein which is substantially homologous to the coding segments in Figure 13, as well as other naturally occurring GGF polypeptides for the purpose of inducing muscle mitogenesis. Also included are the use of: allelic variations; natural mutants; induced mutants; proteins encoded by DNA that hybridizes under high or low stringency conditions to a nucleic acid naturally occurring (for definitions of high and low stringency see *Current Protocols in Molecular Biology*, (1989) John Wiley & Sons, New York, NY, 6.3.1 - 6.3.6, hereby incorporated by reference); and the use of polypeptides or proteins specifically bound by antisera to GGF polypeptides. The term also includes the use of chimeric polypeptides that include the GGF polypeptides comprising sequences from Figure 13.

Use of Neuregulins

A novel aspect of the invention involves the use of neuregulins as factors to promote cell communication by inducing the production of products. These Products affect the function of these cells. Treatment of the cells to achieve these effects may be achieved by contacting cells with a polypeptide described herein.

The methods of the invention may also be used to treat a patient suffering from a disease caused by a lack of trophic factor(s). The lack of trophic factor(s) is defined by a decreased amount of trophic factor(s) relative to that of an unaffected individual sufficient to cause detectable alteration in the biological effect of those trophic factor(s). The neurotrophic factor(s) may be present at levels 10% below those observed in unaffected individuals. More preferably, the factor(s) are present at levels 20% lower than that observed in unaffected individuals, and most preferably the levels are lowered by 80% relative to unaffected individuals under similar circumstances.

The methods of the invention make use of the fact that the neuregulin proteins are encoded by the same gene. A variety of messenger RNA splicing variants (and their resultant proteins) are derived from this gene and many of these products show binding to $p185^{erbB2}$ and related receptors and activation of the same. This invention provides a use for all of the known products of the neuregulin gene (described herein and in the references listed above). Most preferably, recombinant human GGF2 (rhGGF2) is used in these methods.

The invention also relates to the use of other, not yet naturally isolated, splicing variants of the neuregulin gene. Figure 12 shows the known patterns of splicing. These patterns are derived from polymerase chain reaction experiments (on reverse transcribed RNA), analysis of cDNA clones (as presented within), and analysis of published sequences encoding neuregulins (Peles et al., *Cell* (1992) **69**:205 and Wen et al., *Cell* (1992) **69**:559). These patterns, as well as additional patterns disclosed herein, represent probable splicing variants which exist. The splicing variants are fully described in Goodearl et al., USSN 08/036,555, filed March 24, 1993, incorporated herein by reference.

More specifically, effects on cell communication may be achieved by contacting cells with a polypeptide defined by the formula

WYBAZCX

wherein WYBAZCX is composed of the polypeptide segments shown in Figure 13; wherein W comprises the polypeptide segment F, or is absent wherein Y comprises the polypeptide segment E, or is absent; wherein Z comprises the polypeptide segment G or is absent; and wherein X comprises the polypeptide segment C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, or C/D C/D' D' HKL.

Furthermore, the invention includes a method of treating muscle cells by the application to the muscle cell of a

-30kD polypeptide factor isolated from the MDA-MB 231 human breast cell line; or

-35kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line to the glial cell; or

-75kD polypeptide factor isolated from SKBR-3 human breast cell line; or

-44kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line; or

-25kD polypeptide factor isolated from activated mouse peritoneal macrophages; or

-45kD polypeptide factor isolated from the MDA-MB 231 human breast cell; or

-7 to 14kD polypeptide factor isolated from the ATL-2 human T-cell line to the glial cell; or

-25kD polypeptide factor isolated from the bovine kidney cells; or

-42kD ARIA polypeptide factor isolated from brain; or

-46-47kD polypeptide factor which stimulates 0-2A glial progenitor cells; or

-43-45kD polypeptide factor, GGFIII, U.S. patent application Serial No. 07/931,041, filed August 17, 1992, incorporated herein by reference.

The invention includes use of any modifications or equivalents of the above polypeptide factors which do not exhibit a significantly reduced activity. For example, modifications in which amino acid content or sequence is altered without substantially adversely affecting activity are included. The statements of effect and use contained herein are therefore to be construed accordingly, with such uses and effects employing modified or equivalent factors being part of the invention.

The human peptide sequences described above and presented in Figures 13, 14, 15, and 16, respectively, represent a series of splicing variants which can be isolated as full-length complementary DNAs (cDNAs) from natural sources (cDNA libraries

prepared from the appropriate tissues) or can be assembled as DNA constructs with individual exons (e.g., derived as separate exons) by someone skilled in the art.

Another aspect of the invention is the use of a pharmaceutical or veterinary formulation comprising any factor as defined above formulated for pharmaceutical or veterinary use, respectively, optionally together with an acceptable diluent, carrier or excipient and/or in unit dosage form. In using the factors of the invention, conventional pharmaceutical or veterinary practice may be employed to provide suitable formulations or compositions.

A medicament is made by administering the polypeptide with a pharmaceutically effective carrier.

Thus, the formulations to be used as a part of the invention can be applied to parenteral administration, for example, intravenous, subcutaneous, intramuscular, intraorbital, ophthalmic, intraventricular, intracranial, intracapsular, intraspinal, intracisternal, intraperitoneal, topical, intranasal, aerosol, scarification, transdermal and by other slow release devices (i.e., osmotic pump-driven devices; see also U.S.S.N. 08/293,465, hereby incorporated by reference) and also oral, buccal, rectal or vaginal administration.

The formulations of this invention may also be administered by the transplantation into the patient of host cells expressing the DNA encoding polypeptides which are effective for the methods of the invention or by the use of surgical implants which release the formulations of the invention.

Parenteral formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well-known in the art for making formulations are to be found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain as excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes, biocompatible, biodegradable lactide polymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the present factors. Other potentially useful parenteral delivery systems for the factors include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable

infusion systems, and liposomes. Formulations for inhalation may contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration, methoxysalicylate for rectal administration, or citric acid for vaginal administration.

The present factors can be used as the sole active agents, or can be used in combination with other active ingredients, e.g., other growth factors which could facilitate neuronal survival in neurological diseases, or peptidase or protease inhibitors.

The concentration of the present factors in the formulations of the invention will vary depending upon a number of issues, including the dosage to be administered, and the route of administration.

In general terms, the factors of this invention may be provided in an aqueous physiological buffer solution containing about 0.1 to 10% w/v compound for parenteral administration. General dose ranges are from about 1 μg/kg to about 1 g/kg of body weight per day; a preferred dose range is from about 0.01 mg/kg to 100 mg/kg of body weight per day. The preferred dosage to be administered is likely to depend upon the type and extent of progression of the pathophysiological condition being addressed, the overall health of the patient, the make up of the formulation, and the route of administration.

A further general aspect of the invention is the use of a factor of the invention in the manufacture of a medicament, preferably for the treatment of a disease or disorder. The "GGF2" designation is used for all clones which were previously isolated with peptide sequence data derived from GGF-II protein (i.e., GGF2HBS5, GGF2BPP3) and, when present alone (i.e., GGF2 or rhGGF2), to indicate recombinant human protein encoded by plasmids isolated with peptide sequence data derived from the GGF-II protein (i.e., as produced in insect cells from the plasmid HBS5). Recombinant human GGF from the GGFHBS5 clone is called GGF2, rhGGF2 and GGF2HBS5 polypeptide.

Methods for treatment of diseases or disorders using neuregulins in this manner are also part of the invention. Administration of neuregulins to induce the production of a substance or substances from a neuregulin responsive cell can be used in any disorder where an increase in a neuregulin inducible substance that is trophic for the disease

affected neurons would be of benefit. In peripheral nerve injury or peripheral nerve disorders such as the neuropathies administration of neuregulins will elicit the production of neurotrophic substances from known neuregulin target tissues such as Schwann cells and muscle. These induced substances can enhance axonal repair. Alzheimer's disease is another target for neuregulin therapy. In the brain, neuregulins are detectable in cholinergic motor neurons (Chen, et al., *J. Comparative Neurology* (1994) **349**:389-400), these neurons degenerate in Alzheimer's disease and many show trophic responses to neurotrophic factors such as NGF. Neuregulins can be used to induce the synthesis of neurotrophic factors in those neurons that interact with cholinergic neurons. Similar therapeutic approaches may be used in other neurodegenerative disorders such as Parkinson's disease, amyotrophic lateral sclerosis, spinal muscular atrophy or any disease where stimulation of the synthesis of substances that are trophic for disease affected neurons might be of benefit.

Methods for treatment of diseases or disorders using nucleic acid constructs encoding neuregulins or neuregulin producer cells are also part of the invention.

Delivery of DNA to a cell or tissue that will take up the DNA, express the DNA and produce neuregulin as shown by Wolff et al., (*Science* (1990) **247**:1465) and Ascadi et al., (*Nature* (1991) **352**:815) is an aspect of the invention. The neuregulin produced by this method will act on the first cell type and elicit the responses described above. Genetic modification of cultured cells (or their precursors) such as fibroblasts (as shown by Wolff et al. *Proc. Nat'l Acad. Sci. USA* (1988) **86**:1575) or such as those derived from the nervous system (as shown by Weiss et al. International Patent Application number PCT/US94/01053; publication number WO 94/16718) to induce the production of neuregulin from the cultured cells is another aspect of this invention. The genetically modified neuregulin producer cells can be transplanted to a position near the first cell type and elicit the responses described above.

Assays for Determining Neuregulin Effect(s) on Cellular Communication

Described below are generic methods for detecting the ability of a neuregulin to induce in a first cell type, the production of a product (Product A) that is trophic for a second cell type. A general reference on cell and tissue culture is *Cell and Tissue Culture: Laboratory Procedures* (Ed. by A. Doyle, J. B. Griffiths, and D. G. Newell, John Wiley and Sons, New York, NY, 1994). General references on the culture of neural cells and tissues are *Methods in Neurosciences, Vol. 2* (Ed. by P. M. Conn. Academic Press, Sand Diego, CA, 1990) and *Culturing Nerve Cells* (Ed. by G. Banker and K. Goslin, MIT Press, Cambridge, MA 1991). General references of immunocytochemistry are *Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988), and *Immunocytochemistry II* (Ed. by A. C. Cuello, John Wiley and Sons, New York, NY, 1993).

The vertebrate cells used in this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, *Meth. Enz.* (1979) **58**:44; Barnes and Sato, *Anal. Biochem.* (1980) **102**:255; U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195 and U.S. Pat. Re. 30,985, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression and will be apparent to the ordinarily skilled artisan.

Method I -

The use of separate cultures of a first cell type and a second cell type, to demonstrate that neuregulin induces the first cell type to produce a secreted substance that is trophic for the second cell type.

1. Establish cultures of cells from the tissue of interest (e.g. spinal cord, pancreas, gut, etc.). These cultures are enriched for the first cell type such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers (e.g. tubulin β3 for neurons (A. Banerjee, M. C. Roach, P. Trcka, and R. F. Luduena, Increased microtubule assembly in bovine brain tubulin lacking the type III isotype of b tubulin. *J. Biol. Chem.* (1990) **265**:1794-1799), Islet-1 for pancreatic islet cells (O. Karlsson, S. Thor, T. Norbert, H. Ohlsson, and T. Edlund, Insulin gene enhanced binding protein Isl-1 is a member of a novel class of proteins containing both a homeo and a Cys-His domain. *Nature* (1990) **344**:879-882)).

2. Establish cultures of cells from the same tissue of interest as in step 1. These cultures are enriched for the second cell type such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers.

3. Expose the first cell type cultures to varying doses of neuregulin for varying periods of time, preferably greater than 1 minute and less than 7 days. At the end of the culture period, collect the conditioned culture medium, remove debris by centrifugation (200 g, 10 minutes) and filtration (nylon filter, 0.22 mm pore size). This medium (conditioned medium) will contain the secreted product(s) of the first cell type , Product A.

4. Replace or supplement the medium of the second cell type cultures with media prepared as in step 3. Include among these medium samples, medium that has been conditioned by the first cell type cultures in the absence of neuregulin (control conditioned medium). Include among the medium samples, media containing neuregulin that have not been conditioned by the first cell type cultures (non-conditioned medium).

5. Maintain the second cell type cultures as described in step 4 for varying periods of time preferably greater than 1 day and less than 7 days. Assess various aspects of

cellular phenotype such as, but not limited to, cell survival, morphology, production of enzymes and secreted products, etc.

6. Assess the effects of the neuregulin. The neuregulin is trophic for the first cell type in a manner that promotes the production of products trophic for the second cell type if:

a. Medium conditioned by the first cell type cultures in the presence of neuregulin maintains or increases desired aspects of cellular phenotype such as, but not limited to cell survival, morphology, production of enzymes and secreted products, etc.;

b. equal volumes of control conditioned medium lack the activity described in criterion (a.), or demonstrate lesser degrees of the activity described in criterion (a.);

and

c. equal volumes of non-conditioned medium lack the activity described in criterion (a.), or demonstrate lesser degrees of the activity described in criterion (a.).

The induction by neuregulin of a secreted product, Product A, such that Product A affects a third cell type, can also be tested as in Method I. Establish cultures of cells from the same tissue of interest as in step 1. These cultures are enriched for the third cell type, such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers. Substitute the third cell type cultures for the second cell type cultures in steps 4-6.

If Product A is not secreted, but is bound to the surface of the first cell type, or is bound to insoluble extracellular matrix associated with the first cell type, an alternative procedure is to be used:

Method II -

The use of separate cultures of the first and second cell types, to demonstrate that neuregulin induces the first cell type to produce a substance on its surface that is trophic for the second cell type.

1. Establish cultures of cells from the tissue of interest (e.g. spinal cord, pancreas, gut, etc.). These cultures are enriched for the first cell type such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers (e.g. tubulin β3 for neurons, Islet-1 for pancreatic islet cells).

2. Expose the first cell type cultures to varying doses of neuregulin for varying periods of time, preferably greater than 1 hour and less than 7 days. At the end of the culture period, remove the culture medium and establish a co-culture of the first and second cell types as follows. Rinse the first cell type cultures 3 times with fresh culture medium lacking neuregulin so as to rinse away residual neuregulin. Add back a suspension of the second cell type, from the same tissue of interest as in step 1 in fresh medium lacking neuregulin. The suspension is enriched for the second cell type, such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers.

3. In parallel to step 2, plate the same suspension of cells of the second cell type on the first cell type cultures that have not been treated with neuregulin (control co-cultures).

4. Maintain the first cell type/second cell type co-cultures for varying periods of time preferably greater than 1 day and less than 7 days. Assess various aspects of cellular phenotype of the second cell type such as, but not limited to, cell survival, morphology, production of enzymes and secreted products, etc.

5. Assess the effects of neuregulin. Neuregulin is trophic for the first cell type in a manner that promotes the production of products trophic for the second cell type if:

a. The first cell type cultures pre-treated with neuregulin maintain or increase desired aspects of cellular phenotype of the second cell type such as, but not limited to cell survival, morphology, production of enzymes and secreted products, etc.;

and

b. The first cell type cultures that have not been pre-treated with Product A lack the activity described in criterion (a.), or demonstrate lesser degrees of the activity described in criterion (a.).

The induction by neuregulin of a cell surface-bound or extracellular matrix-bound product, Product A, such that Product A affects a third cell type, can also be tested as in Method II. In steps 2-4, use a suspension of the third cell type rather than the second cell type such that preferably greater than 90% of the cells can be demonstrated to be the third cell type through the use of immunocytochemical and/or enzymatic markers.

Described below are methods for detecting the activities of a neuregulin that induces neuronally-associated tissues to produce a neurotrophic product or product(s) (Product A):

Method III -

The use of separate cultures of neurons and neuronally associated tissues, to demonstrate that neuregulin induces a neuronally associated tissue (the first cell type) to produce a secreted product that is trophic for neurons (the second cell type).

1. Establish neuron-free cultures of neuronally-associated cell types (e.g. glia, fibroblasts). These cultures are enriched for a single cell type (the first cell type) such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers (e.g. S-100 for peripheral glia (K. R. Jessen and R. Mirsky, Schwann cell: early lineage, regulation of proliferation and control of myelin formation. *Curr. Op. Neurobiol.* (1992) **2**:575-581), fibronectin for fibroblasts (K. M. Yamada, Cell surface interactions with extracellular materials. *Ann. Rev. Biochem.* (1983) **52**:761-799)).

2. Establish cultures of neurons from the neuronal tissue of interest (e.g. superior cervical ganglion, spinal motor column). These cultures are enriched for neurons (the second cell type) such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers (e.g. tubulin β3 for all neurons, choline acetyltransferase for cholinergic neurons (J.C. Martinou, A. L. V. Thai, G. Cassar, F. Roubinet, and M. J. Weber, Characterization of two factors enhancing choline acetyltransferase in cultures of purified rat motoneurons. *J. Neurosci.* (1989) **9**:3645-3656)).

3. Expose the first cell type cultures to varying doses of neuregulin for varying periods of time, preferably greater than 1 hour and less than 7 days. At the end of the culture period, collect the conditioned culture medium, remove debris by centrifugation (200 g, 10 minutes) and filtration (nylon filter, 0.22 mm pore size).

4. Replace or supplement the medium of the second cell type cultures with media prepared as in step 3. Include among these medium samples, medium that has been conditioned by the first cell type cultures in the absence of neuregulin (control conditioned medium). Include among the medium samples, media containing neuregulin that have not been conditioned by the first cell type cultures (non-conditioned medium).

5. Maintain the second cell type cultures as described in step 4 for varying periods of time preferably greater than 1 day and less than 7 days. Assess various aspects of

neuronal phenotype such as, but not limited to cell survival, neurite (axon or dendrite) outgrowth, neurotransmitter phenotype, etc.

6. Assess the effects of neuregulin. Neuregulin is trophic for neuronally-associated tissues in a manner that promotes the production of neurotrophic products if:

a. Medium conditioned by the first cell type cultures in the presence of neuregulin maintains or increases desired aspects of neuronal phenotype such as, but not limited to cell survival, increased neurite (axon or dendrite) outgrowth, neurotransmitter synthesis, etc.;

b. equal volumes of control conditioned medium lack the activity described in criterion (a.), or demonstrate lesser degrees of the activity described in criterion (a.);

and

c. equal volumes of non-conditioned medium lack the activity described in criterion (a.), or demonstrate lesser degrees of the activity described in criterion (a.).

If Product A is not secreted, but is bound to the surface of the first cell type, or is bound to insoluble extracellular matrix associated with the first cell type, an alternative procedure is to be used:

Method IV -

The use of separate cultures of neurons and neuronally associated tissues, to demonstrate that neuregulin induces a neuronally associated tissue (the first cell type) to produce a substance on its surface that is trophic for neurons.

1. Establish neuron-free cultures of neuronally-associated cell types (e.g. glia, fibroblasts). These cultures are enriched for a single cell type (the first cell type) such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers (e.g. S-100 for peripheral glia, fibronectin for fibroblasts).

2. Expose the first cell type cultures to varying doses of neuregulin for varying periods of time, preferably greater than 1 hour and less than 7 days. At the end of the culture period, remove the culture medium and establish a co-culture of the first cell type and neurons (the second cell type) as follows. Rinse the first cell type cultures 3 times with fresh culture medium lacking neuregulin so as to rinse away residual neuregulin. Add back a suspension of neurons from the neuronal tissue of interest (e.g. superior cervical ganglion, spinal motor column) in fresh medium lacking neuregulin. The suspension is enriched for the second cell type such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers (e.g. tubulin β3 for all neurons, choline acetyltransferase for cholinergic neurons).

3. In parallel to step 2, plate the same suspension of the second cell type cells on the first cell type cultures that have not been treated with Product A (control co-cultures).

4. Maintain the first cell type/second cell type co-cultures for varying periods of time preferably greater than 1 day and less than 7 days. Assess various aspects of neuronal phenotype such as, but not limited to cell survival, neurite (axon or dendrite) outgrowth, neurotransmitter phenotype, etc.

5. Assess the effects of neuregulin. Neuregulin is trophic for the first cell type in a manner that promotes the production of products trophic for the second cell type if:

a. The first cell type cultures pre-treated with neuregulin maintain or increase desired aspects of neuronal phenotype such as, but not limited to cell survival, neurite (axon or dendrite) outgrowth, neurotransmitter phenotype, etc.;

and

b. The first cell type cultures that have not been pre-treated with Product A lack the activity described in criterion (a.), or demonstrate lesser degrees of the activity described in criterion (a.).

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If cultures of non-neuronal cells of interest greater than 90% pure have not been established, the following method can be used:

Method V -

The use of a mixed culture, to demonstrate that neuregulins induce the first cell type (neuronally associated cell types) to produce a product (Product A) that affects the second cell type.

1. Establish undissociated, explant cultures of the neuronal tissue of interest (e.g. superior cervical ganglion, spinal motor column). These cultures are not enriched for various cell types and are constituted of both neurons (the second cell type) and neuronally-associated cell types (the first cell type) as demonstrated through the use of immunocytochemical and/or enzymatic markers (e.g. tubulin β3 for all neurons, acetylcholinesterase for cholinergic neurons, S-100 for peripheral glia, fibronectin for fibroblasts).

2. Expose explant cultures to varying doses of neuregulin for varying periods of time, preferably greater than 1 hour and less than 7 days. At the end of the culture period, assess various aspects of neuronal phenotype such as, but not limited to neuron survival, neurite (axon or dendrite) outgrowth, neurotransmitter phenotype, etc.

3. Establish cultures of neurons from the neuronal tissue of interest (e.g. superior cervical ganglion, spinal motor column). These cultures are enriched for neurons (the second cell type) such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers (e.g. tubulin β3 for all neurons, choline acetyltransferase for cholinergic neurons).

4. Expose the second cell type cultures to varying doses of neuregulin for varying periods of time, preferably greater than 1 hour and less than 7 days. At the end of the culture period, assess various aspects of neuronal phenotype such as, but not limited to neuron survival, neurite (axon or dendrite) outgrowth, neurotransmitter phenotype, etc.

5. Assess the effects of neuregulin. Neuregulin is trophic for neuronally-associated tissues in a manner that promotes the production of neurotrophic products if:

a. in explant cultures the presence of neuregulin maintains or increases desired aspects of neuronal phenotype such as, but not limited to neuron survival, neurite (axon or dendrite) outgrowth, neurotransmitter synthesis, etc.;

and

b. in the second cell type cultures, neuregulin lacks the activity described in criterion (a.), or demonstrates lesser degrees of the activity described in criterion (a.)

EXAMPLES

Example 1

The Effect of Recombinant Human Glial Growth Factor 2 on Sympathetic Ganglion Outgrowth in an *In Vitro* Model of Peripheral Nerve Gap Entubulation

Purpose

One approach to the repair of injuries in which a peripheral nerve has been severed is to suture the nerve endings together via a biocompatible tube, a procedure referred to as entubulation. The tube may be filled with various agents thought to improve the growth and regeneration of the nerve. Peripheral nerves contain a variety of cell types: neurons (or more appropriately, the axons emanating from neuron cell bodies located in the spinal cord and associated ganglia), Schwann cells (peripheral glia), fibroblasts, and resident macrophages. Axons regenerate from the side of the nerve gap proximal to the spinal cord and associated ganglia; other cell types contribute to regeneration by migrating in from both sides of the gap and proliferating.

In an effort to devise an *in vitro* model of entubulation, a technique was developed in which fragments of the rat superior cervical ganglion (SCG) are cultured in segments of surgical tubing used in whole animal models of peripheral nerve entubulation. SCG neurons are homogenous in their trophic requirements and project axons exclusively through peripheral nerves; SCG fragments also contain Schwann cells, fibroblasts, and macrophages. In this model the SCG fragments serve as surrogate proximal nerve endings, and the outgrowth of axons and supporting cell types can be observed in a simplified environment. The focus of this example was to examine the effects of rhGGF2 on Schwann cell and axon behavior in this *in vitro* model of peripheral nerve entubulation.

Methods and Materials

Tube Preparation

Tubing used for this study was polyethylene tubing with an internal diameter of 1.19 mm and outer diameter of 1.70 mm (Intramedic®: Becton Dickinson and Company; Parsippany, New Jersey). A length of tubing somewhat longer than actually needed was cut in a sterile tissue culture hood, immersed in 70% ethanol, and flushed repeatedly with 70% ethanol using a syringe with a 19-gauge needle. After soaking the

tubing for approximately 30 minutes, it was flushed again with air, and allowed to dry in the hood. After drying, the tubing was cut into 10 mm segments with a sterile scalpel, and stored in a sterile Petri dish.

Culture Medium

Culture medium was made freshly on the day of culture assembly. All components were kept cold (either 4° C or on ice), as was the final solution until culture assembly was completed. .

Sterile Water	2.60
Sodium bicarbonate (2% w/v)	1.50
Penicillin/Streptomycin stock*	0.15
L-Glutamine (200 mM)	0.15
Fetal Bovine Serum**	0.75
Sodium hydroxide (0.1 M)	0.90
10x Medium***	1.50
Collagen solution****	7.40
TOTAL	15.00 ml

* 5000 units/ml penicillin, 5 mg/ml streptomycin

** heat inactivated (Hyclone; Logan, UT)

*** one packet of low glucose Dulbecco's Modified Essential Medium (DMEM: Gibco/BRL; Grand Island, NY) meant to make 1 liter of medium dissolved in 100 ml of sterile water

**** 3 mg/ml Vitrogen-100® (Celtrix Pharmaceuticals; Santa Clara, CA)

Medium was used as is, or was supplemented with rhGGF2 as indicated.

Tube Culture Assembly

A schematic diagram of culture assembly is shown in Figure 1. SCGs were dissected from postnatal day 0-2 rats on the day of assembly, cleaned of connective tissues and proximal nerve stumps, bisected, and stored in physiological saline at 4° till needed. Since the collagen-containing medium gels at room temperature or higher, it is necessary to assemble the cultures in 4° cold room. Working with watchmaker's forceps under a dissection microscope at total magnification of 8x, individual segments of cleaned tubing were picked up and filled with culture medium using a syringe with a 27-gauge needle. A single piece of bisected SCG was then placed at the very end of each tube, and each tube placed in an individual well of a 24-well tissue culture plate. Only the central eight wells of a 24-well plate were used for tube placement, and the remaining wells were filled with sterile water to maintain plate humidity. The plate was then placed in a 37°, 10% carbon dioxide incubator. After allowing the cultures to gel and equilibrate with the incubator atmosphere, the plates were sealed with paraffin film to further protect the culture assemblies from dehydration, and returned to the incubator until preparation for immunocytochemistry and analysis.

Immunocytochemistry

After 2, 5, and 10 days *in vitro*, the contents of individual tube cultures were extruded into phosphate buffered saline (PBS) using a PBS-filled syringe with a blunt-ended 18-gauge needle. The collagen gels retained structural integrity and were fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature. After 3 washes with PBS, the cultures were blocked in 1% goat serum/0.1% Triton X-100 in PBS for 30 minutes. After blocking, the solution was changed to 1% goat serum in PBS (GPBS) containing 1:4 rabbit anti-S-100 (a Schwann cell marker; Incstar; Stillwater, MN) and 1:400 mouse anti-tubulin ß3 (an axon marker; Sigma; St. Louis, MO). After incubating the samples in primary antibody for 1 hour at room temperature, they were washed 3 times with PBS, and incubated for an additional hour in GPBS containing 1:200 peroxidase-conjugated goat anti-mouse immunoglobulin, and 1:200 alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Pierce Chemical; Rockford, IL). The samples were then washed 3 times with PBS, and the stains developed. The S-100 was first developed using stable pre-mixed NBT/BCIP (Gibco/BRL) to yield a blue stain, and after rinsing with PBS, the tubulin β3 was developed using 3-amino-9-ethylcarbazole (AEC; Sigma) per manufacturer's directions. After final rinsing with PBS, the samples were mounted on microscope slides using aqueous mounting medium. After the mounting medium dried, the cellular outgrowth could be analyzed.

Scoring and Analysis

As schematized in Figure 2, a grid reticule was placed in the microscope ocular, and at a total magnification of 160x, the total number of S-100+ Schwann cells in each column (referred to as "bins") was counted. Each bin has a width of 50 mm as determined using a stage micrometer. And as noted, the number of tubulin β3+ neurites intersecting every vertical line was also counted. The actual grid was not large enough to cover the entire length of cellular outgrowth and was shifted along as needed by translational movement of the microscope stage. All data points represent the average ± the standard error of the mean (n = 6 to 7 for every data point).

Results

First shown is the analysis of Schwann cell number as a function of distance from the SCG explant (Figure 3A-D). It is clear that the presence of rhGGF2 affects the behavior of Schwann cells relative to the control condition. There does not appear to be any difference among the 3 doses of rhGGF2. Generally, by 5 days in rhGGF2, there is a large increase in the number of Schwann cells proximal to the explant, but the Schwann cells appear to have moved only about as far as they have in the control case (somewhat further at the highest dose). By 10 days in rhGGF2 the overall number of Schwann cells has decreased, but the cells still present have definitely migrated farther than in the absence of rhGGF2. In the absence of rhGGF2, the controls look no different between days 5 and 10. The total number of Schwann cells in the various conditions is shown in Figure 4. Again, there is a decrease in cell number at day 10, but there is no obvious difference between the different doses of rhGGF2. The day 10 tubes contain more debris, and this is probably due to cell death. This is due to the culture situation since 10 days appears to be the longest that one can maintain these tube cultures without overt signs of dehydration and nutrient depletion in the limited volume of culture medium (approximately 10 µl per tube).

A difference is apparent when neurites are scored in the various doses of rhGGF2 (Figure 5). At doses of rhGGF2 greater than or equal to 50 ng/ml, a profound increase takes place in the number of neurites and the extent to which they have grown away from the explant.

Discussion and Conclusions

This study demonstrates that the dose range in which there are observable effects on Schwann cell proliferation and emigration from the explant is different from that which causes a major increase in neurite outgrowth. In the case of the former, it

appears that the effect has plateaued at the lowest dose tested, 5 ng/ml. As for the requirement of ≥50 ng/ml rhGGF2 to boost neurite regeneration, there are two possible mechanisms to account for this. One is that rhGGF2 is acting directly upon the neurons, and the other is that rhGGF2 induces a non-neuronal cell type to produce a neurite promoting factor (e.g. NGF, secreted extracellular matrix proteins, proteases, and/or protease inhibitors). As is demonstrated in Example 2, the first hypothesis is not likely since rhGGF2 has no effect upon neuronal survival or outgrowth in low density cultures of dissociated SCG neurons. This lack of a direct effect on neurons implies that the rhGGF2 promotion of neurite outgrowth is due to rhGGF2 induced production of neurite promoting factors by non-neuronal cells.

Example 2

The Promotion of Axon Outgrowth by Recombinant Human Glial Growth Factor 2 is Not Due to a Direct Effect on Neurons

Purpose

As demonstrated in Example 1, rhGGF2 not only promotes Schwann cell proliferation and migration in an *in vitro* model of peripheral nerve entubulation, but also promotes robust axonal outgrowth. To test whether this may be due to direct effects of rhGGF2 on SCG neurons, low density cultures of dissociated SCG neurons were established in which the effects of rhGGF2 could be examined. SCG neurons are normally dependent upon nerve growth factor (NGF) for survival, so rhGGF2 was tested for direct neuronal effects in the simultaneous presence of a wide range of NGF concentrations.

Methods and Materials

Cell Culture

SCGs were dissected from postnatal day 0-2 rats, cleaned of connective tissue and proximal nerve stumps, and dissociated by enzymatic digestion and trituration. Enzymatic digestion was performed using 1 mg/ml trypsin (Sigma; St. Louis, MO) and 1 mg/ml collagenase (Boehringer-Mannheim; Indianapolis, IN) in calcium- and magnesium-free Hanks's Balanced Salt Solution (HBSS; Gibco/BRL; Grand Island, NY), for 1 hour at 37° C. Trituration was performed using a flame-polished Pasteur pipet. Dissociated neurons were taken up in plating medium and pre-plated in tissue culture dishes for 1 hour to remove the majority of the rapidly adherent, non-neuronal cells. Plating medium consisted of low glucose DMEM (Gibco/BRL) supplemented with glutamine, penicillin/streptomycin, and fetal bovine serum to the same concentrations as described in Example 1. Non-adherent cells (primarily neurons), were pelleted by centrifugation and resuspended in plating medium. These cells were finally plated at a density of 5000 cells per well in collagen-coated, 24-well plates such that the cells were exposed to a 2-dimensional dose-response matrix of NGF and rhGGF2 (Figure 6A). Plates were set up in duplicate on 2 different dates; at the completion of both experiments N = 4 for each of the 24 conditions. The cultures were only allowed to progress for 2 days since this is a time frame in which any

contaminating Schwann cells could have only undergone a single doubling, and sufficient for ascertaining whether the factors have promoted neuronal survival.

Staining and Scoring of the Cultures

After 2 days, the cultures were fixed and stained for tubulin β3 as described in Example 1. The tubulin β3-positive, neurite-bearing cells were counted in each well at a total magnification of 100x. Due to meniscus effects, and incubator vibration during the initial plating period, cells tend to preferentially concentrate in the center of the well. Thus in order to get a reasonably representative count of cell number, 5 fields per well were counted: the center most field and four flanking fields (Figure 6B). This manner of counting was used on all wells and is sufficiently consistent for the purpose of comparing the effects of different growth factor concentrations and combinations. The number of cells counted in every well was normalized such that the average number counted in the wells that received 0 ng/ml rhGGF2, and 100 ng/ml NGF equals a value of 100.

Results

It is clear from the results presented in Figure 7, that rhGGF2 has no direct effect on the survival of SCG neurons. All surviving neurons exhibited robust axon outgrowth, and there was no noticeable effect on the extent of axon outgrowth. As expected in the absence of rhGGF2, the number of neurons reaches a plateau at 10 ng/ml. The presence or absence of rhGGF2 appears to make no difference at the 3 doses tested.

Discussion and Conclusions

In light of the results presented in Example 1, it was necessary to examine whether the effect of rhGGF2 on axon outgrowth could be attributed to a direct effect of rhGGF2 on the neurons in question. The results of Example 2 make it clear that this is not the case. Thus one must conclude that the effect of rhGGF2 on axon outgrowth observed in the tube paradigm is due to a "bystander effect" rather than a direct action on the neurons. Thus rhGGF2 can promote the healing response of injured neurons by inducing the production of neurite promoting factors by non-neuronal support cells.

Example 3

Increase in myelinated axon growth in an animal model of peripheral nerve injury mediated by a neuregulin

An animal model of peripheral nerve repair was used to test the ability of a neuregulin (rhGGF2) to increase the number of regenerating axons. The rationale is that added rhGGF2 will induce increases in Schwann cell (the first cell type) numbers as well as increases in the levels of trophic factors (Product A) produced by Schwann cells that, in turn, will affect a second cell type, the regenerating axons (the second cell type) as measured by increases in the number of myelinated axons (response).

Fisher 344 rats (male, 195-250g) were surgically prepared and one sciatic nerve was transected resulting in a 10mm gap. Polyethylene guide tubes (13mm in length, 1.1mm internal diameter) were prepared. These tubes contained a flat sliver of a collagen coated Immobilon filter (1.0x10mm) containing immobilized rhGGF2 and were prepared as described in U.S. Patent Application Serial No. 08/293,465, filed on August 19, 1994, hereby incorporated by reference (Immobilon: Millipore, Corp., Bedford, MA). The strips were inserted into the lumen of the guide tubes. rhGGF2 was used at a concentration of 162 μg/μL (in phosphate buffered saline), 2.5 μL of this solution was added per strip. Control tubes were prepared containing collagen coated Immobilon strips treated with phosphate buffered saline alone. Tubes were secured with a single suture at the proximal and distal ends after filling the lumen with physiological saline and sealing the ends with vaseline.

Animals (10 rhGGF2 treated, 10 controls) were sacrificed at 28 days and the section of sciatic nerve containing the tube was excised, the nerve was removed from the tube and a cross section was taken from the mid point of the tube and prepared for histological analysis. The material was fixed in 4% paraformaldehyde and 2% glutaraldehyde for 24h and then post fixed in 2% osmium tetroxide and embedded in glycomethacrylate One micron cross sections were taken and stained with 1μM toluidine blue.

A histological analysis of a section from the mid point of the tube was performed and measurements were made of the total number of myelinated axons in a section and the total endoneurial area in each section. The data are shown in Figures 8A and 8B.

The rhGGF2 treated animals showed a 2.1 fold increase in the number of myelinated axons over the control animals.

The results of this study demonstrate a positive effect of exogenously added rhGGF2 on the growth of myelinated axons. In consideration of the data discussed in Example 1 where rhGGF2 acts on Schwann cells to induce the synthesis of products that are trophic for regenerating axons in an *in vitro* paradigm it is concluded that a similar mechanism is responsible for the rhGGF2 mediated enhancement of the growth of axons *in vivo*.

Claims:

1. A method of affecting cellular communication in a vertebrate, comprising administration of a neuregulin to said vertebrate wherein said neuregulin interacts with a first cell type, resulting in production of a Product A of said first cell type and said Product A affects a function of a second cell type.

2. A method of Claim 1, wherein, said affect in function of said second cell type results in the production of a Product B of said second cell type and said Product B affects the function of said first cell type or a third cell type.

3. A method of affecting cellular communication in a vertebrate, comprising administration of a neuregulin-producing cell to said vertebrate wherein a neuregulin is produced and said neuregulin interacts with a first cell type, resulting in production of a Product A of said first cell type and said Product A affects a function of a second cell type.

4. A method of Claim 3, wherein, said affect in function of said second cell type results in the production of a Product B of said second cell type and said Product B affects the function of said first cell type or a third cell type.

5. A method of affecting cellular communication in a vertebrate, comprising administration of DNA encoding a neuregulin to said vertebrate wherein DNA is incorporated into a genome of a cell and said DNA is expressed in said cell resulting in the production of said neuregulin which interacts with a first cell type, resulting in production of a Product A of said first cell type and said Product A affects a function of a second cell type.

6. A method of Claim 5, wherein, said affect in function of said second cell type results in the production of a Product B of said second cell type and said Product B affects the function of said first cell type or a third cell type.

7. A method of Claim 1 wherein said vertebrate is a human.

8. A method of Claim 1 wherein said first cell type is a nervous system support cell.

9. A method of Claim 8 wherein said nervous system support cell is a Schwann cell.

10. A method of Claim 1 wherein said first cell type is a neuron.

11. A method of Claim 1 wherein said first cell type is a muscle cell.

12. A method of Claim 1 wherein said Product A is a neurotrophic agent.

13. A method of Claim 1 wherein said Product A is a matrix molecule.

14. A method of Claim 1 wherein said Product A is a protease.

15. A method of Claim 1 wherein said Product A is a protease inhibitor.

16. A method of Claim 1 wherein said second cell type is a nervous system cell.

17. A method of Claim 1 wherein said second cell type is a muscle cell.

18. A method of Claim 1 wherein said affect in function of said second cell type is differentiation.

19. A method of Claim 1 wherein said affect in function of said second cell type is mitosis.

20. A method of Claim 1 wherein said affect in function of said second cell type is survival.

21. A method of Claim 2 wherein said Product B is a neurotrophic agent.

22. A method of Claim 2 wherein said Product B is a matrix molecule.

23. A method of Claim 2 wherein said Product B is a protease.

24. A method of Claim 2 wherein said Product B is a protease inhibitor

25. A method of Claim 2 wherein said Product B is a neuregulin.

26. A method of Claim 25 wherein said neuregulin is rhGGF2.

27. A method of Claim 2 wherein said affect in function of said first cell type or said third cell type is differentiation.

28. A method of Claim 2 wherein said affect in function of said first cell type or said third cell type is mitosis.

29. A method of Claim 2 wherein said affect in function of said first cell type or said third cell type is survival.

30. A method of Claim 2 wherein said third cell type is a nervous system cell.

31. A method of Claim 2 wherein said third cell type is a muscle cell.

32. A method of treating a neurological disorder in a mammal, comprising administration of a therapeutically effective amount of a neuregulin to said mammal wherein said neuregulin interacts with a nervous system cell, resulting in the production of a neurotrophic agent which affects the function of a neuron cell type.

33. A method of treating a neurological disorder in a mammal, comprising administration of a neuregulin producing cell to said mammal wherein said produced neuregulin interacts with a nervous system cell, resulting in the production of a neurotrophic product which affects the function of a neuron cell type.

34. A method of treating a neurological disorder in a mammal, comprising administration of DNA encoding a neuregulin to said mammal wherein said neuregulin is produced and interacts with a nervous system cell, resulting in the production of a neurotrophic agent which affects the function of a neuron cell type.

35. A method of treating peripheral neuropathy, amyotrophic lateral sclerosis, spinal muscular atrophy, nerve injury, Alzheimer's Disease, Parkinson's Disease and spinal cord injury comprising the administration of a therapeutically effective amount of a neuregulin wherein said neuregulin interacts with a first cell type, resulting in the production of a Product A of the first cell type and said Product A affects a function of a second cell type.

36. A method of inducing the endogenous production of a product by a cell in a vertebrate comprising administration of a neuregulin to said vertebrate, wherein said cell produces said product.

37. A method of claim 36 wherein said product is a neurotrophic agent.

Figure 1

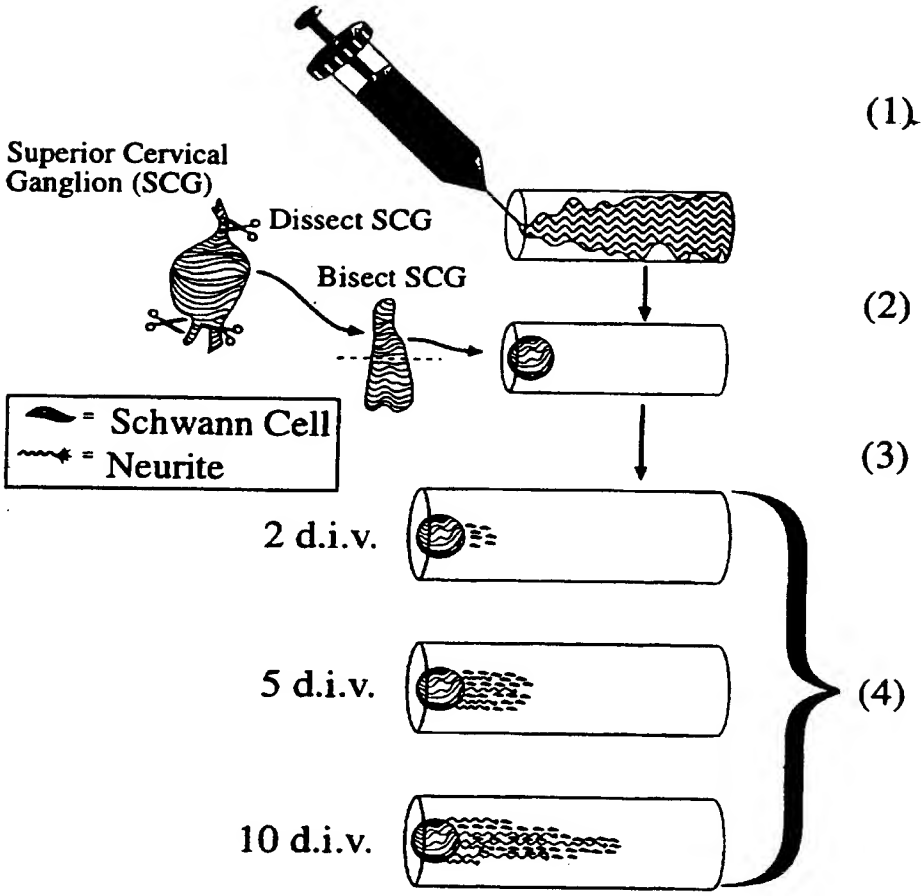


Figure 2

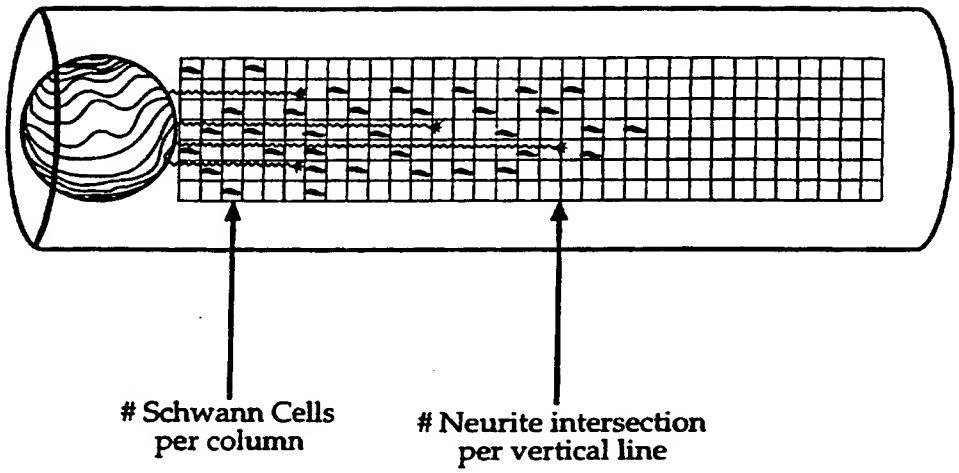


Figure 3A

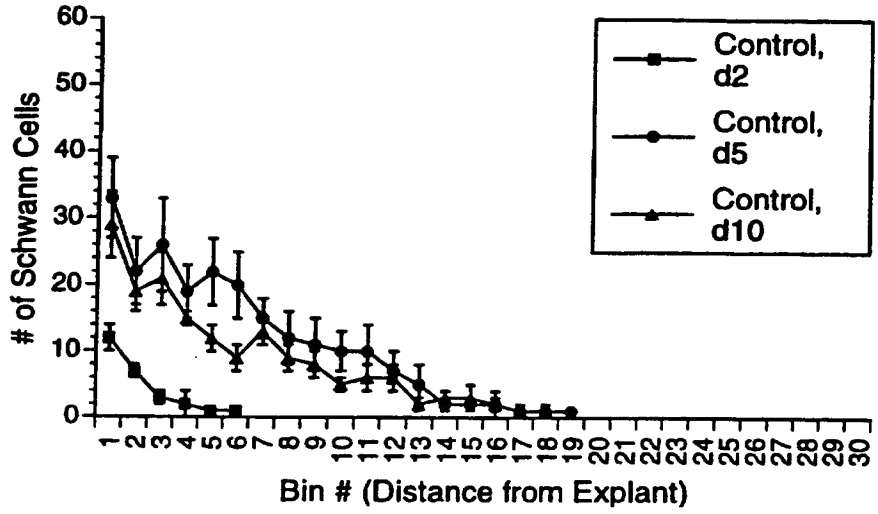


Figure 3B

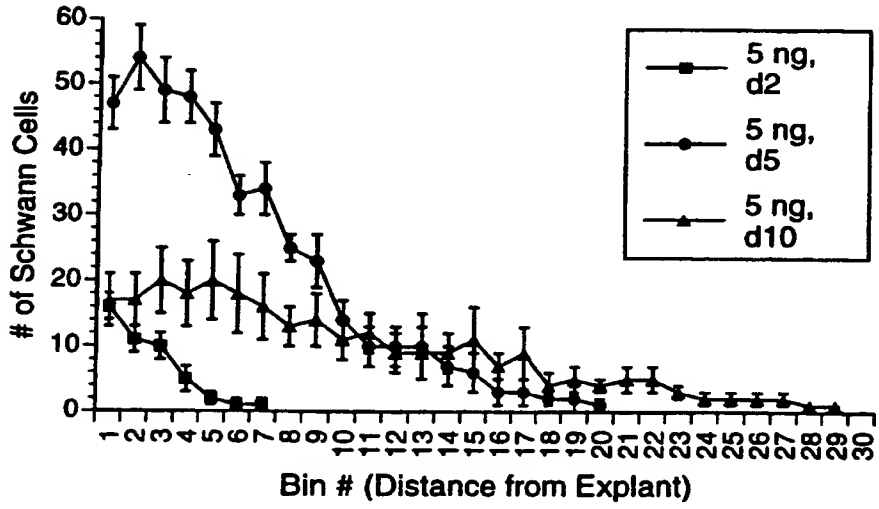


Figure 3C

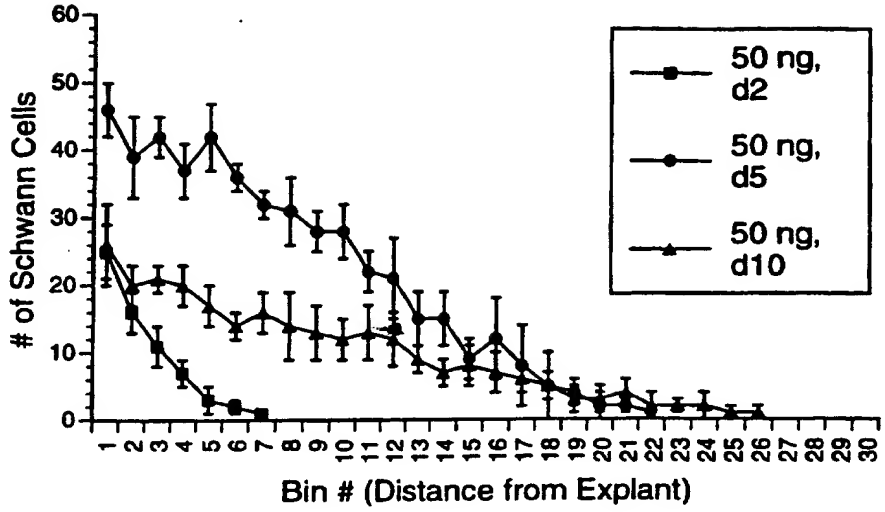


Figure 3D

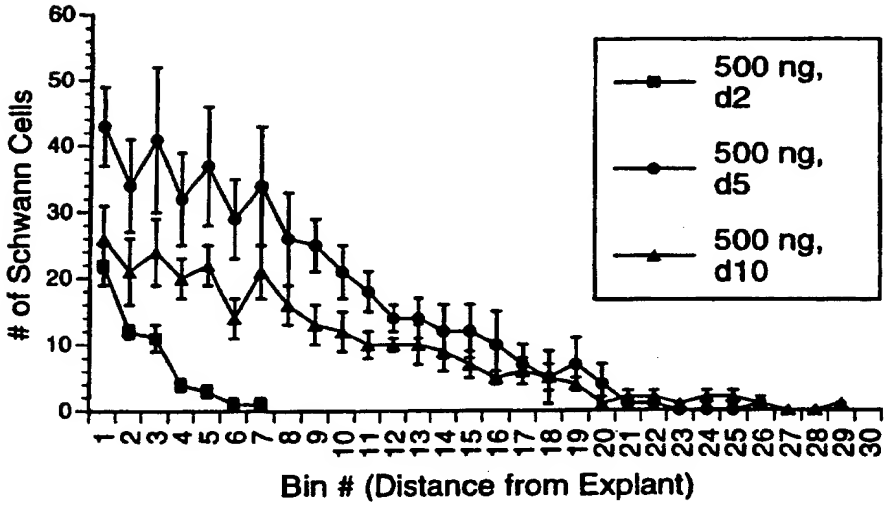


Figure 4

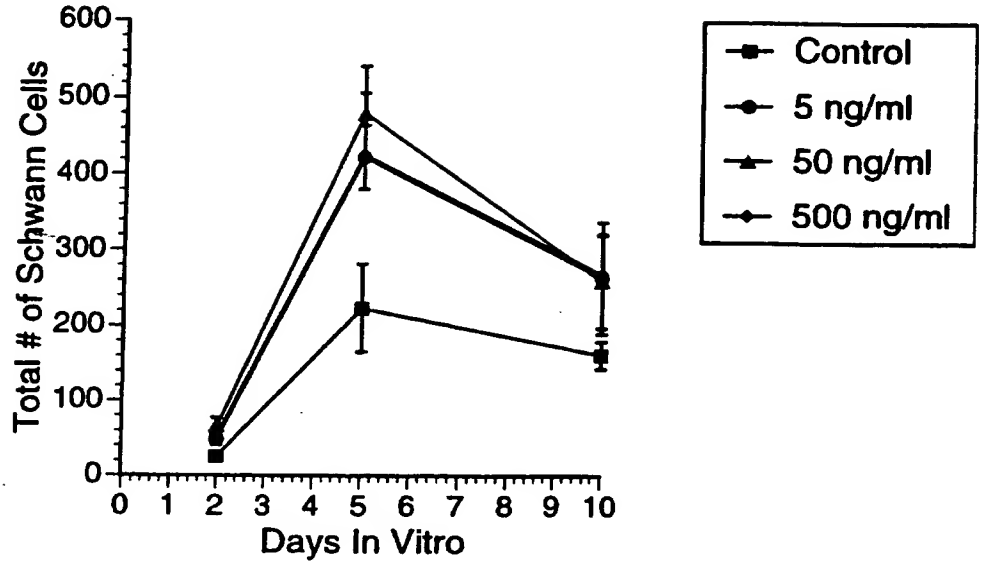


Figure 5

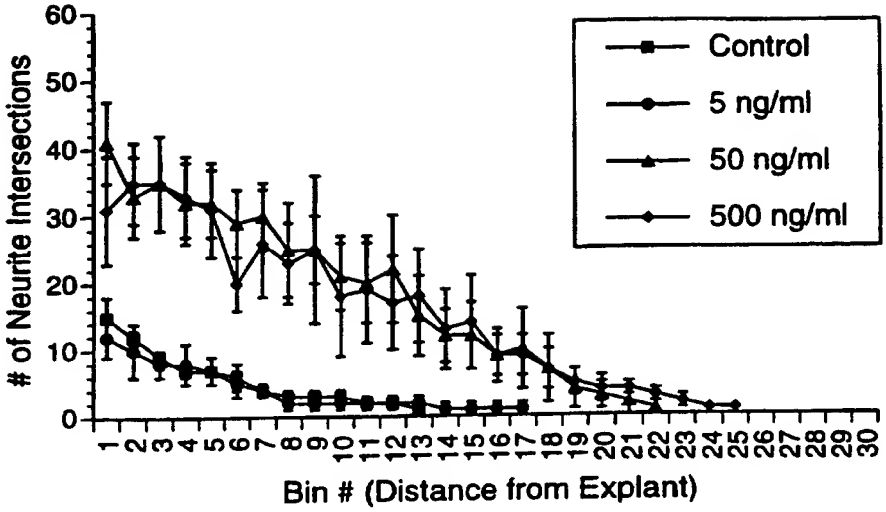


Figure 6A

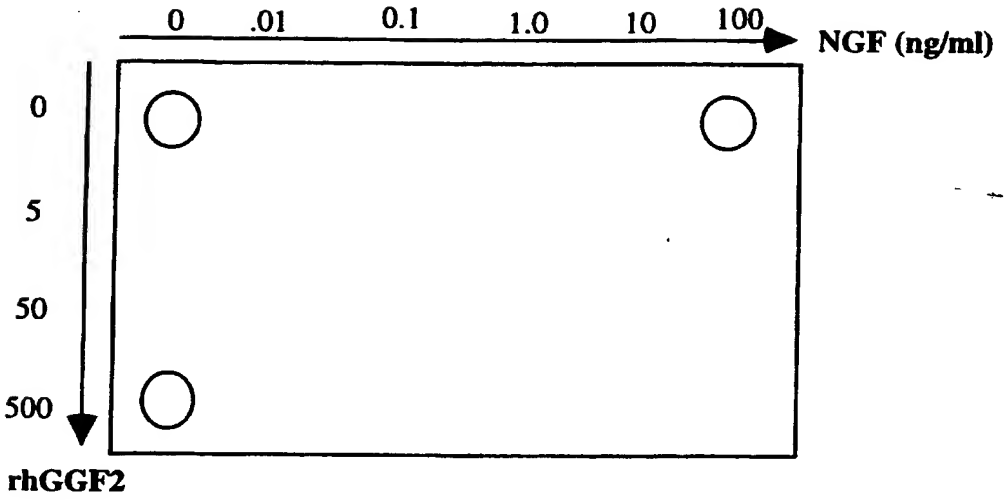


Figure 6B

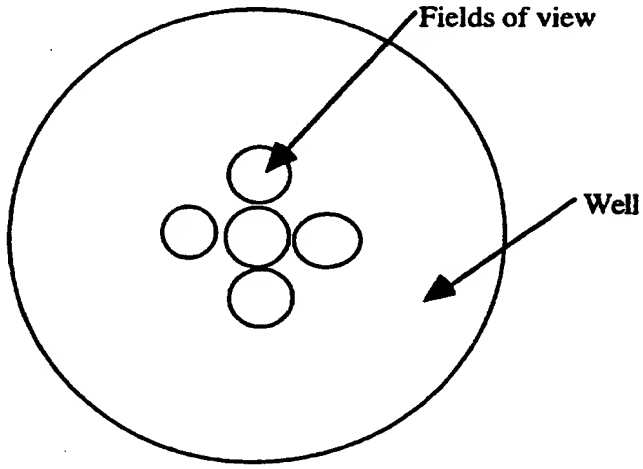


Figure 7

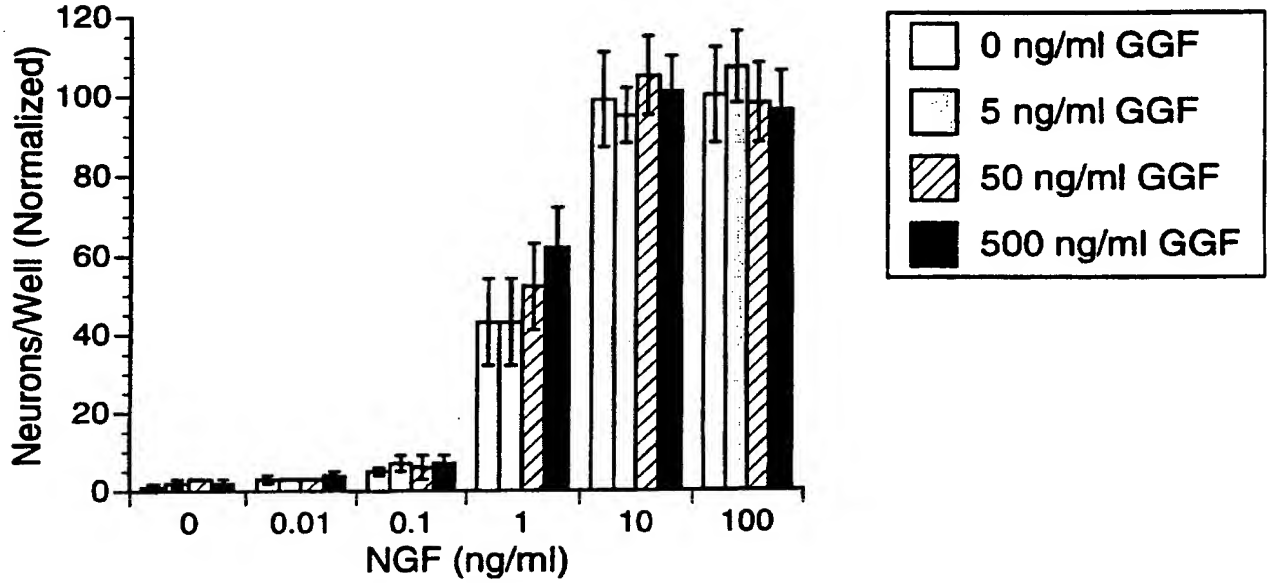


Figure 8A

	Total Myelinated Axons	Total Endoneurial Area	Axon Density
GGF group (n=9)			
GGF2 mean	741	165345(μM^2)	4143(per mm^2)
GGF2 std. error	200	14809	951
Control group (n=10)			
Control mean	355	130931(μM^2)	2938(per mm^2)
Control std. error	76	14917	722

Figure 8B

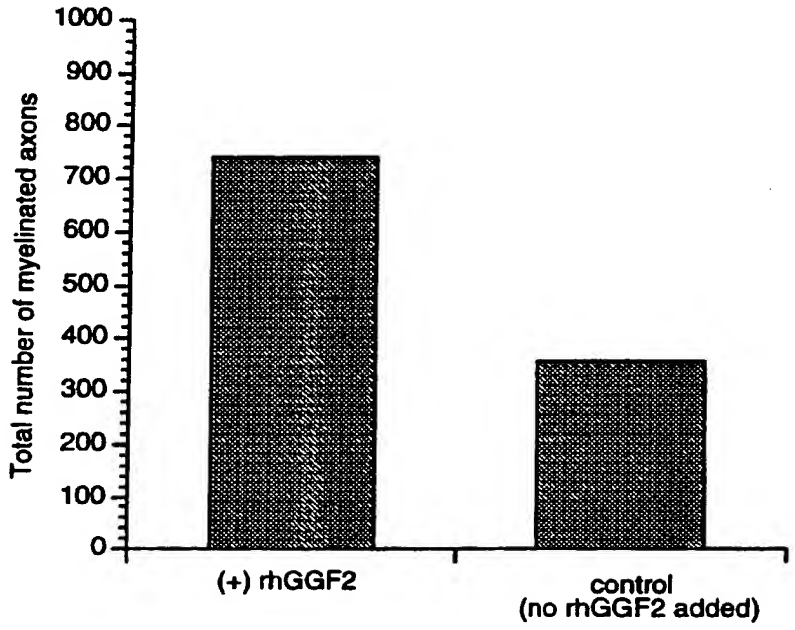
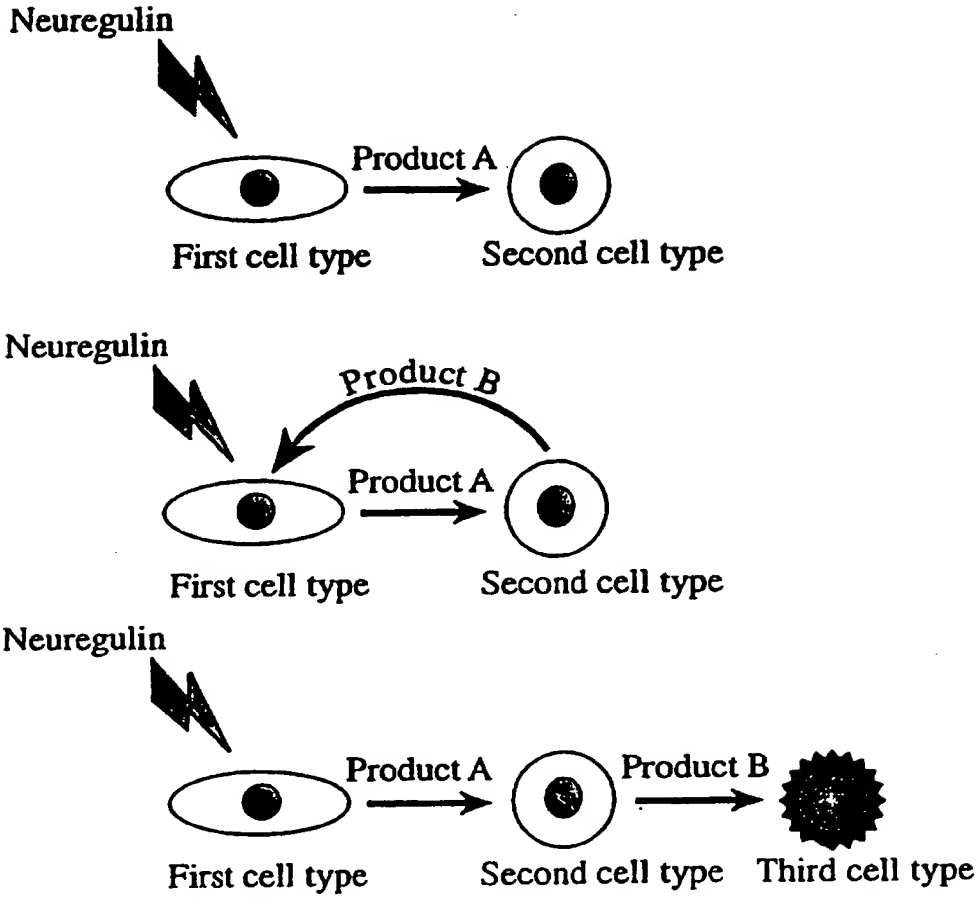


Figure 9



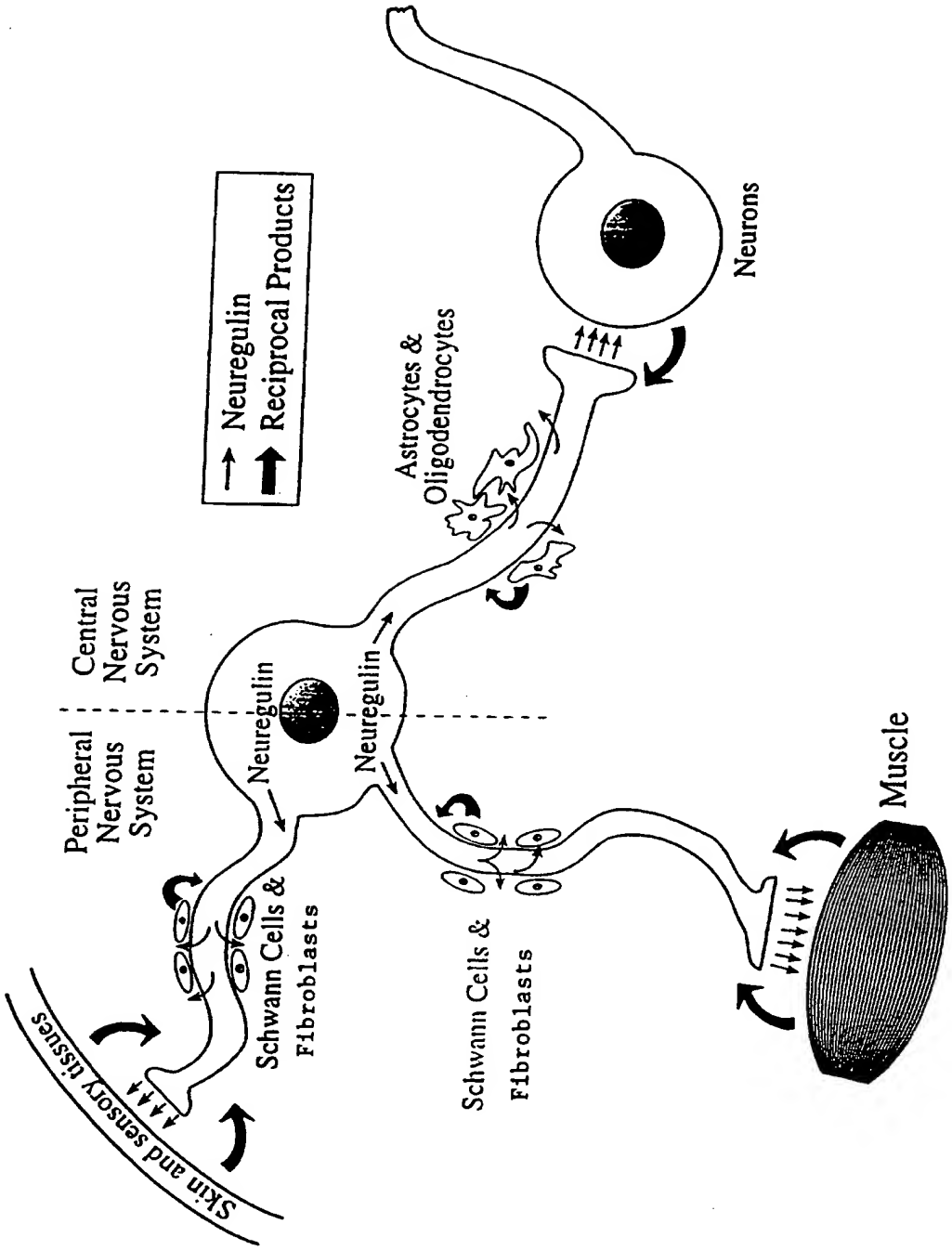


Figure 10

Figure 11 A

```
CCTGCAG CAT CAA GTG TGG GCG GCG AAA GCC GGG GGC TTG AAG AAG GAC TCG CTG      55
        His Gln Val Trp Ala Ala Lys Ala Gly Gly Leu Lys Lys Asp Ser Leu
          1               5                  10                  15

CTC ACC GTG CGC CTG GGC GCC TGG GGC CAC CCC GCC TTC CCC TCC TGC          103
Leu Thr Val Arg Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser Cys
            20                  25                  30

GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC GAG          151
Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro Glu
        35                  40                  45
GCC AAC AGC AGC GGC GGG CCC GGC CGC CTT CCG AGC CTC CTT CCC CCC          199
Ala Asn Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro Pro
    50                  55                  60

TCT CGA GAC GGG CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT GTG          247
Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Gln Pro Gly Ala Val
65                  70                  75                  80

CAA CGG TGC GCC TTG CCT CCC CGC TTG AAA GAG ATG AAG AGT CAG GAG          295
Gln Arg Cys Ala Leu Pro Pro Arg Leu Lys Glu Met Lys Ser Gln Glu
                85                  90                  95

TCT GTG GCA GGT TCC AAA CTA GTG CTT CGG TGC GAG ACC AGT TCT GAA          343
Ser Val Ala Gly Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu
            100                 105                 110

TAC TCC TCT CTC AAG TTC AAG TGG TTC AAG AAT GGG AGT GAA TTA AGC          391
Tyr Ser Ser Leu Lys Phe Lys Trp Phe Lys Asn Gly Ser Glu Leu Ser
        115                 120                 125

CGA AAG AAC AAA CCA GAA AAC ATC AAG ATA CAG AAA AGG CCG GGG AAG          439
Arg Lys Asn Lys Pro Glu Asn Ile Lys Ile Gln Lys Arg Pro Gly Lys
    130                 135                 140

TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA TAT          487
Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr
145                 150                 155                 160

ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC          535
Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn
                165                 170                 175

ATC ACC ATT GTG GAG TCA AAC GGT AAG AGA TGC CTA CTG CGT GCT ATT          583
Ile Thr Ile Val Glu Ser Asn Gly Lys Arg Cys Leu Leu Arg Ala Ile
            180                 185                 190

TCT CAG TCT CTA AGA GGA GTG ATC AAG GTA TGT GGT CAC ACT                  625
Ser Gln Ser Leu Arg Gly Val Ile Lys Val Cys Gly His Thr
        195                 200                 205

TGAATCACGC AGGTGTGTGA AATCTCATTG TGAACAAATA AAAATCATGA AAGGAAAAAA        685

AAAAAAAAAA AATCGATGTC GACTCGAGAT GTGGCTGCAG GTCGACTCTA GAGGATCCC         744
```

Figure 11 B

```
CCTGCAG CAT CAA GTG TGG GCG GCG AAA GCC GGG GGC TTG AAG AAG GAC TCG CTG      55
        His Gln Val Trp Ala Ala Lys Ala Gly Gly Leu Lys Lys Asp Ser Leu
        1               5                   10                  15

CTC ACC GTG CGC CTG GGC GCC TGG GGC CAC CCC GCC TTC CCC TCC TGC          103
Leu Thr Val Arg Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser Cys
            20                  25                  30

GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC GAG          151
Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro Glu
        35                  40                  45

GCC AAC AGC AGC GGC GGG CCC GGC CGC CTT CCG AGC CTC CTT CCC CCC          199
Ala Lys Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro Pro
    50                  55                  60

TCT CGA GAC GGG CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT GTG          247
Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Gln Pro Gly Ala Val
65                  70                  75                  80

CAA CGG TGC GCC TTG CCT CCC CGC TTG AAA GAG ATG AAG AGT CAG GAG          295
Gln Arg Cys Ala Leu Pro Pro Arg Leu Lys Glu Met Lys Ser Gln Glu
                85                  90                  95

TCT GTG GCA GGT TCC AAA CTA GTG CTT CGG TGC GAG ACC AGT TCT GAA          343
Ser Val Ala Gly Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu
            100                 105                 110

TAC TCC TCT CTC AAG TTC AAG TGG TTC AAG AAT GGG AGT GAA TTA AGC          391
Tyr Ser Ser Leu Lys Phe Lys Trp Phe Lys Asn Gly Ser Glu Leu Ser
        115                 120                 125

CGA AAG AAC AAA CCA GAA AAC ATC AAG ATA CAG AAA AGG CCG GGG AAG          439
Arg Lys Asn Lys Gly Gly Asn Ile Lys Ile Gln Lys Arg Pro Gly Lys
    130                 135                 140

TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA TAT          487
Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr
145                 150                 155                 160

ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC          535
Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn
                165                 170                 175
```

Figure 11 B'

```
ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG ACA          583
Ile Thr Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly Thr
        180                 185                 190

AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT          631
Ser His Leu Val Lys Ser Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
    195                 200                 205

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC          679
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
210                 215                 220

TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT          727
Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn
225                 230                 235                 240

GTG CCC ATG AAA GTC CAA ACC CAA GAA AGT GCC CAA ATG AGT TTA CTG          775
Val Pro Met Lys Val Gln Thr Gln Glu Ser Ala Gln Met Ser Leu Leu
                245                 250                 255

GTG ATC GCT GCC AAA ACT ACG TAATGGCCAG CTTCTACAGT ACGTCCACTC             826
Val Ile Ala Ala Lys Thr Thr
            260

CCTTTCTGTC TCTGCCTGAA TAGCGCATCT CAGTCGGTGC CGCTTTCTTG TTGCCGCATC       886

TCCCCTCAGA TTCCTCCTAG AGCTAGATGC GTTTTACCAG GTCTAACATT GACTGCCTCT       946

GCCTGTCGCA TGAGAACATT AACACAAGCG ATTGTATGAC TTCCTCTGTC CGTGACTAGT      1006

GGGCTCTGAG CTACTCGTAG GTGCGTAAGG CTCCAGTGTT TCTGAAATTG ATCTTGAATT      1066

ACTGTGATAC GACATGATAG TCCCTCTCAC CCAGTGCAAT GACAATAAAG GCCTTGAAAA      1126

GTCAAAAAAA AAAAAAAAAA AAAAATCGA TGTCGACTCG AGATGTGGCT GCAGGTCGAC       1186

TCTAGAG                                                                1193
```

Figure 11 C

```
CCTGCAG CAT CAA GTG TGG GCG GCG AAA GCC GGG GGC TTG AAG AAG GAC TCG CTG      55
        His Gln Val Trp Ala Ala Lys Ala Gly Gly Leu Lys Lys Asp Ser Leu
        1               5                   10                  15

CTC ACC GTG CGC CTG GGC GCC TGG GGC CAC CCC GCC TTC CCC TCC TGC          103
Leu Thr Val Arg Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser Cys
            20                  25                  30

GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC GAG          151
Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro Glu
        35                  40                  45

GCC AAC AGC AGC GGC GGG CCC GGC CGC CTT CCG AGC CTC CTT CCC CCC          199
Ala Asn Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro Pro
    50                  55                  60

TCT CGA GAC GGG CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT GTG          247
Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Gln Pro Gly Ala Val
65                  70                  75                  80

CAA CGG TGC GCC TTG CCT CCC CGC TTG AAA GAG ATG AAG AGT CAG GAG          295
Gln Arg Cys Ala Leu Pro Pro Arg Leu Lys Glu Met Lys Ser Gln Glu
                85                  90                  95

TCT GTG GCA GGT TCC AAA CTA GTG CTT CGG TGC GAG ACC AGT TCT GAA          343
Ser Val Ala Gly Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu
            100                 105                 110

TAC TCC TCT CTC AAG TTC AAG TGG TTC AAG AAT GGG AGT GAA TTA AGC          391
Tyr Ser Ser Leu Lys Phe Lys Trp Phe Lys Asn Gly Ser Glu Leu Ser
        115                 120                 125

CGA AAG AAC AAA CCA GAA AAC ATC AAG ATA CAG AAA AGG CCG GGG AAG          439
Arg Lys Asn Lys Pro Glu Asn Ile Lys Ile Gln Lys Arg Pro Pro Lys
    130                 135                 140

TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA TAT          487
Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr
145                 150                 155                 160
```

Figure 11 C'

```
ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC                535
Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn
                165                 170                 175

ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG ACA               583
Ile Arg Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly Thr
            180                 185                 190

AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT               631
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
        195                 200                 205

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC               679
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
    210                 215                 220

TTG TGC AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC               727
Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr
225                 230                 235                 240

GTA ATG GCC AGC TTC TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT               775
Val Met Ala Ser Phe Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro
                245                 250                 255

GAA TAGCGCATCT CAGTCGGTGC CGCTTTCTTG TTGCCGCATC TCCCCTCAGA TTCCGCCTAG        838
Glu

AGCTAGATGC GTTTTACCAG GTCTAACATT GACTGCCTCT GCCTGTCGCA TGAGAACATT            898

AACACAAGCG ATTGTATGAC TTCCTCTGTC CGTGACTAGT GGGCTCTGAG CTACTCGTAG            958

GTGCGTAAGG CTCCAGTGTT TCTGAAATTG ATCTTGAATT ACTGTGATAC GACATGATAG           1018

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Figure 12

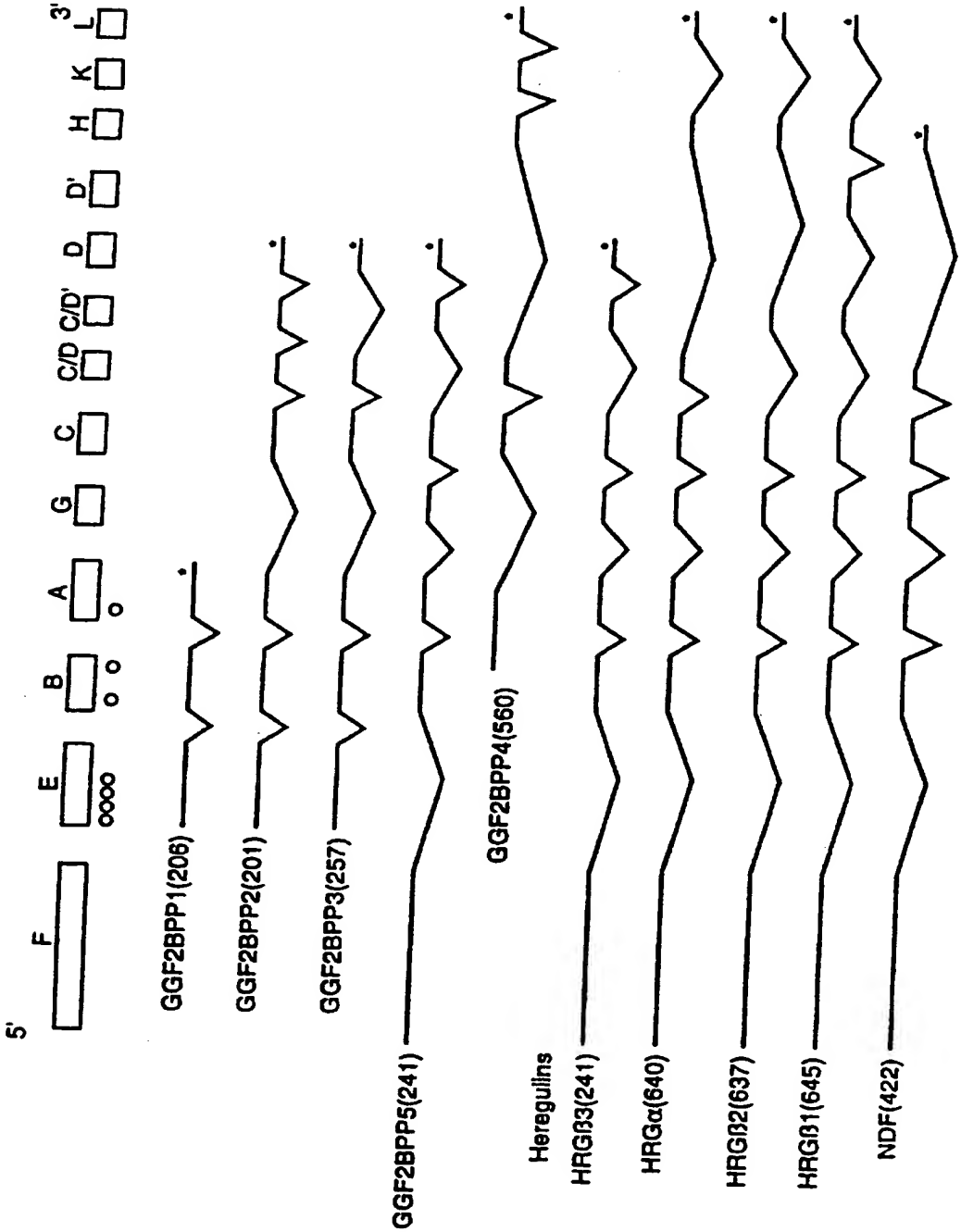

Figure 13 A

CODING SEGMENT F:

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TGCGAGCGCG CCGGACCGAG GCAGCGACAG GAGCGGACCG CGGCGGGAAC CGAGGACTCC     180

CCAGCGGCGC GCCAGCAGGA GCCACCCCGC GAGNCGTGCG ACCGGGACGG AGCGCCCGCC     240

AGTCCCAGGT GGCCCGGACC GCACGTTGCG TCCCCGCGCT CCCCGCCGGC GACAGGAGAC     300

GCTCCCCCCC ACGCCGCGCG CGCCTCGGCC CGGTCGCTGG CCCGCCTCCA CTCCGGGGAC     360
                |||| | |||||| ||  ||| |||| | | | | ||  |||  |||||
                CGCGAG CGCCTCAGCG CGGCCGCTCG CTCTC..CCC CTCGAGGGAC

AAACTTTTCC CGAAGCCGAT CCCAGCCCTC GGACCCAAAC TTGTCGCGCG TCGCCTTCGC     420
|||||||||| | || ||||| || |||||| |||||  ||                |||||| |||
AAACTTTTCC CAAACCCGAT CCGAGCCCTT GGACCAAA.. .........C TCGCCTGCGC

                                          Met Ser Glu Arg Arg
CGGGAGCCGT CCGCGCAGAG CGTGCACTTC TCGGGCGAG ATG TCG GAG CGC AGA     474
|| |||||||  |||||  ||||  ||   | | ||  || |||||||  ||| ||  |||  ||| | |
CGAGAGCCGT CCGCGTAGAG CGCTC.CGTC TCCGGCGAG ATG TCC GAG CGC AAA
                                                          K

Glu Gly Lys Gly Lys Gly Lys Gly Gly Lys Lys Asp Arg Gly Ser Gly
GAA GGC AAA GGC AAG GGG AAG GGC GGC AAG AAG GAC CGA GGC TCC GGG     522
|||  |||  | |  |||  ||  |||  |||  |||        |||  |||  ||  |||  |||  ||
GAA GGC AGA GGC AAA GGG AAG GGC AAG AAG AAG GAG CGA GGC TCC GGC
        R                      K           E

Lys Lys Pro Val Pro Ala Ala Gly Gly Pro Ser Pro Ala
AAG AAG CCC GTG CCC GCG GCT GGC GGC CCG AGC CCA G     559
|||  |||  ||  |  |  ||  |||  ||  |||  ||  |  |||  |||  |
AAG AAG CCG GAG TCC GCG GCG GGC AGC CAG AGC CCA G
          E   S
```

Figure 13 B

CODING SEGMENT E:

```
CC CAT CAN GTG TGG GCG GCG AAA GCC GGG GGC TTG AAG AAG GAC TCG          47
   His Gln Val Trp Ala Ala Lys Ala Gly Gly Leu Lys Lys Asp Ser
     1               5                  10                  15

CTG CTC ACC GTG CGC CTG GGC GCC TGG GGC CAC CCC GCC TTC CCC TCC          95
Leu Leu Thr Val Arg Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser
                20                  25                  30

TGC GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC         143
Cys Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro
            35                  40                  45

GAG GCC AAC AGC AGC GGC GGG CCC GGC CGC CTT CCG AGC CTC CTT CCC         191
Glu Ala Asn Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro
        50                  55                  60

CCC TCT CGA GAC GGG CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT         239
Pro Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Gln Pro Gly Ala
    65                  70                  75

GTG CAA CGG TGC G                                                   252
Val Gln Arg Cys
 80
```

Figure 13 C

CODING SEGMENT B:

```
Leu Pro Pro Arg Leu Lys Glu His Lys Ser Gln Glu Ser Val Ala Gly
CCT TGC CTC CCC GCT TGA AAG AGA TGA AGA GTC AGG AGT CTG TGG CAG          48
||| ||| ||| ||| | | ||| ||| ||| ||| | | | ||| | | | | ||| |||
CCT TGC CTC CCC GAT TGA AAG AGA TGA AAA GCC AGG AAT CGG CTG CAG
            Q                                       A

Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu Tyr Ser Ser Leu
GTT CCA AAC TAG TGC TTC GGT GCG AGA CCA GTT CTG AAT ACT CCT CTC          96
||| ||| ||| ||| | | ||| ||| | | | | ||| ||| ||| ||| ||| ||| |||
GTT CCA AAC TAG TCC TTC GGT GTG AAA CCA GTT CTG AAT ACT CCT CTC

Lys Phe Lys Trp Phe Lys Asn Gly Ser Glu Leu Ser Arg Lys Asn Lys
TCA AGT TCA AGT GGT TCA AGA ATG GGA GTG AAT TAA GCC GAA AGA ACA         144
|||   | ||| ||| ||| ||| ||| ||| ||| ||  ||| | |   | ||| | | |||
TCA GAT TCA AGT GGT TCA AGA ATG GGA ATG AAT TGA ATC GAA AAA ACA
R                           N           N

Pro Gly Asn Ile Lys Ile Gln Lys Arg Pro Gly
AAC CAC AAA ACA TCA AGA TAC AGA AAA GGC CGG G                        178
||| ||| ||| | | ||| ||| ||| | | |||  || | | |
AAC CAC AAA ATA TCA AGA TAC AAA AAA AGC CAG G
                        K
```

Figure 13 D

CODING SEGMENT A:

```
  Lys Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly
G AAG TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA          46
  ||| ||| ||| ||| ||| ||| | | ||| || ||| ||| ||| ||| ||| |||
G AAG TCA GAA CTT CGC ATT AAC AAA GCA TCA CTG GCT GAT TCT GGA
                        N

Glu Tyr Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser
GAA TAT ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT          94
||  ||| ||| ||| ||| ||| ||| ||| |||  || ||| ||| ||| ||| ||| |||
GAG TAT ATG TGC AAA GTG ATC AGC AAA TTA GGA AAT GAC AGT GCC TCT

Ala Asn Ile Thr Ile Val Glu Ser Asn Ala
GCC AAC ATC ACC ATT GTG GAG TCA AAC G                                 122
||| ||  ||| ||| ||  ||| ||  ||| ||| |
GCC AAT ATC ACC ATC GTG GAA TCA AAC G
```

Figure 13 E

CODING SEGMENT A':

```
TCTAAAACTA CAGAGACTGT ATTTTCATGA TCATCATAGT TCTGTGAAAT ATACTTAAAC      60

CGCTTTGGTC CTGATCTTGT AGG AAG TCA GAA CTT CGC ATT AGC AAA GCG         110
                          Lys Ser Glu Leu Arg Ile Ser Lys Ala
                            1               5

TCA CTG GCT GAT TCT GGA GAA TAT ATG TGC AAA GTG ATC AGC AAA CTA       158
Ser Leu Ala Asp Ser Gly Glu Tyr Met Cys Lys Val Ile Ser Lys Leu
 10                  15                  20                  25

GGA AAT GAC AGT GCC TCT GCC AAC ATC ACC ATT GTG GAG TCA AAC GGT       206
Gly Asn Asp Ser Ala Ser Ala Asn Ile Thr Ile Val Glu Ser Asn Gly
                30                  35                  40

AAG AGA TGC CTA CTG CGT GCT ATT TCT CAG TCT CTA AGA GGA GTG ATC       254
Lys Arg Cys Leu Leu Arg Ala Ile Ser Gln Ser Leu Arg Gly Val Ile
            45                  50                  55

AAG GTA TGT GGT CAC ACT TGAATCACGC AGGTGTGTGA AATCTCATTG              302
Lys Val Cys Gly His Thr
        60

TGAACAAATA AAAATCATGA AAGGAAAACT CTATGTTTGA AATATCTTAT GGGTCCTCCT     362

GTAAAGCTCT TCACTCCATA AGGTGAAATA GACCTGAAAT ATATATAGAT TATTT          417
```

Figure 13 F

CODING SEGMENT G:

```
Glu Ile Thr Thr Gly Met Pro Ala Ser Thr Glu Thr Ala Tyr Val Ser
 AG ATC ACC ACT GGC ATG CCA GCC TCA ACT GAG ACA GCG TAT GTG TCT        47
 || ||| | | ||| ||  ||| ||| ||| ||| ||   |  ||  ||| ||| |||
 AG ATC ATC ACT GGT ATG CCA GCC TCA ACT GAA GGA GCA TAT GTG TCT
            I                                G

Ser Glu Ser Pro Ile Arg Ile Ser Val Ser Thr Glu Gly Thr Asn Thr
TCA GAG TCT CCC ATT AGA ATA TCA GTA TCA ACA GAA GGA ACA AAT ACT      95
||| ||| ||| ||| ||| ||| ||| ||| ||| ||  ||| ||| |||  ||  ||| |||
TCA GAG TCT CCC ATT AGA ATA TCA GTA TCC ACA GAA GGA GCA AAT ACT
                                                    A

Ser Ser Ser
TCT TCA T                                                          102
||| ||| |
TCT TCA T
```

Figure 13 G

CODING SEGMENT C:

```
   Thr Ser Thr Ser Thr Ala Gly Thr Ser His Leu Val Lys Cys Ala
CC ACA TCC ACA TCT ACA GCT GGG ACA AGC CAT CTT GTC AAG TGT GCA      47
|  ||| ||  ||| ||  ||   ||  ||| ||| ||| ||| ||| ||  ||  ||| ||
CT ACA TCT ACA TCC ACC ACT GGG ACA AGC CAT CTT GTA AAA TGT GCG
                       T

Glu Lys Glu Lys Thr Phe Cys Val Asn Gly Gly Glu Cys Phe Met Val
GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGC GAG TGC TTC ATG GTG      95
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||  ||| ||| ||| ||| |||
GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGG GAG TGC TTC ATG GTG

Lys Asp Leu Ser Asn Pro Ser Arg Tyr Leu Cys
AAA GAC CTT TCA AAT CCC TCA AGA TAC TTG TGC                         128
||| ||| ||| ||| ||  ||| ||  ||| ||| ||| |||
AAA GAC CTT TCA AAC CCC TCG AGA TAC TTG TGC
```

Figure 13 H

CODING SEGMENT C/D:

```
Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn Val Pro
AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT GTG CCC      48
||| ||| ||| ||| ||| ||| ||| ||| ||  ||| ||| ||| ||| ||| ||| |||
AAG TGC CAA CCT GGA TTC ACT GGA GCA AGA TGT ACT GAG AAT GTG CCC

Met Lys Val Gln Thr Gln Glu
ATG AAA GTC CAA ACC CAA GAA                                        69
||| ||| ||| ||| | | ||| |||
ATG AAA GTC CAA AAC CAA GAA
              N
```

Figure 13 I

CODING SEGMENT C/D':

```
Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Met
AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC GTA ATG    48
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC GTA ATG

Ala Ser Phe Tyr
GCC AGC TTC TAC                                                    60
||| ||| ||| |||
GCC AGC TTC TAC
```

Figure 13 J

CODING SEGMENT D:

```
Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro Glu  *
AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT GAA TAG                     36
||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT GAA TAG
```

Figure 13 K

CODING SEGMENT D':

```
Lys His Leu Gly Ile Glu Phe Met Glu
AAG CAT CTT GGG ATT GAA TTT ATG GAG                                 27
```

Figure 13 L

CODING SEGMENT H:

```
Lys Ala Glu Glu Leu Tyr Gln Lys Arg Val Leu Thr Ile Thr Gly Ile
AAA GCG GAG GAG CTC TAC CAG AAG AGA GTG CTC ACC ATT ACC GGC ATT        48
||| ||| ||| ||| ||  ||| ||| ||| ||| ||| ||  ||| ||  ||| ||| ||
AAA GCG GAG GAG CTG TAC CAG AAG AGA GTG CTG ACC ATA ACC GGC ATC

Cys Ile Ala Leu Leu Val Val Gly Ile Met Cys Val Val Val Tyr Cys
TGC ATC GCG CTG CTC GTG GTT GGC ATC ATG TGT GTG GTG GTC TAC TGC        96
||| ||| ||  ||  ||  ||| ||  ||| ||| ||| ||| ||| ||| | | ||| |||
TGC ATC GCC CTC CTT GTG GTC GGC ATC ATG TGT GTG GTG GCC TAC TGC
                                           A

Lys Thr Lys Lys Gln Arg Lys Lys Leu His Asp Arg Leu Arg Gln Ser
AAA ACC AAG AAA CAA CGG AAA AAG CTT CAT GAC CGG CTT CGG CAG AGC       144
||| ||| ||| ||| ||  ||| ||| ||| ||  ||| ||| ||  ||| ||| ||| |||
AAA ACC AAG AAA CAG CGG AAA AAG CTG CAT GAC CGT CTT CGG CAG AGC

Leu Arg Ser Glu Arg Asn Thr Met Met Asn Val Ala Asn Gly Pro His
CTT CGG TCT GAA AGA AAC ACC ATG ATG AAC GTA GCC AAC GGG CCC CAC       192
||| ||| ||| ||| ||  ||| |   ||| ||| |||  |  ||| ||  ||| ||  |||
CTT CGG TCT GAA CGA AAC AAT ATG ATG AAC ATT GCC AAT GGG CCT CAC
                        N               I

His Pro Asn Pro Pro Pro Glu Asn Val Gln Leu Val Asn Gln Tyr Val
CAC CCC AAT CCG CCC CCC GAG AAC GTG CAG CTG GTG AAT CAA TAC GTA       240
||  ||  ||  ||  ||| ||| ||| ||  ||  ||| ||| ||| ||| ||| ||| |||
CAT CCT AAC CCA CCC CCC GAG AAT GTC CAG CTG GTG AAT CAA TAC GTA

Ser Lys Asn Val Ile Ser Ser Glu His Ile Val Glu Arg Glu Ala Glu
TCT AAA AAT GTC ATC TCT AGC GAG CAT ATT GTT GAG AGA GAG GCG GAG       288
||| ||| ||  ||| ||| ||  ||  ||| ||| ||| ||| ||| ||| ||  ||  |||
TCT AAA AAC GTC ATC TCC AGT GAG CAT ATT GTT GAG AGA GAA GCA GAG
```

Figure 13 L'

```
Ser Ser Phe Ser Thr Ser His Tyr Thr Ser Thr Ala His His Ser Thr
AGC TCT TTT TCC ACC AGT CAC TAC ACT TCG ACA GCT CAT CAT TCC ACT      336
|   ||  ||| ||| ||| ||| ||| ||  ||| ||  ||| ||  ||| ||  ||| |||
ACA TCC TTT TCC ACC AGT CAC TAT ACT TCC ACA GCC CAT CAC TCC ACT
T

Thr Val Thr Gln Thr Pro Ser His Ser Trp Ser Asn Gly His Thr Glu
ACT GTC ACT CAG ACT CCC AGT CAC AGC TGG AGC AAT GGA CAC ACT GAA      384
||| ||| ||  ||| ||| ||  ||  ||| ||| ||| ||| ||  ||| ||| ||| |||
ACT GTC ACC CAG ACT CCT AGC CAC AGC TGG AGC AAC GGA CAC ACT GAA

Ser Ile Ile Ser Glu Ser His Ser Val Ile Val Met Ser Ser Val Glu
AGC ATC ATT TCG GAA AGC CAC TCT GTC ATC GTG ATG TCA TCC GTA GAA      432
|||  ||| ||  ||  ||| ||| ||| ||| ||  ||| ||| ||| ||| ||| ||| |||
AGC ATC CTT TCC GAA AGC CAC TCT GTA ATC GTG ATG TCA TCC GTA GAA
         L

Asn Ser Arg His Ser Ser Pro Thr Gly Gly Pro Arg Gly Arg Leu Asn
AAC AGT AGG CAC AGC AGC CCG ACT GGG GGC CCG AGA GGA CGT CTC AAT      480
||| ||| ||| ||| ||| ||| ||  ||| ||| ||| ||  ||| ||| ||| ||  |||
AAC AGT AGG CAC AGC AGC CCA ACT GGG GGC CCA AGA GGA CGT CTT AAT

Gly Leu Gly Gly Pro Arg Glu Cys Asn Ser Phe Leu Arg His Ala Arg
GGC TTG GGA GGC CCT CGT GAA TGT AAC AGC TTC CTC AGG CAT GCC AGA      528
|||    ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
GGC ACA GGA GGC CCT CGT GAA TGT AAC AGC TTC CTC AGG CAT GCC AGA
    T

Glu Thr Pro Asp Ser Tyr Arg Asp Ser Pro His Ser Glu Arg
GAA ACC CCT GAC TCC TAC CGA GAC TCT CCT CAT AGT GAA AG              569
||| ||| ||| ||  ||| ||| ||| ||| ||| ||| ||| ||| ||| ||
GAA ACC CCT GAT TCC TAC CGA GAC TCT CCT CAT AGT GAA AG
```

Figure 13 M

CODING SEGMENT K:

```
A CAT AAC CTT ATA GCT GAG CTA AGG AGA AAC AAG GCC CAC AGA TCC          46
  His Asn Leu Ile Ala Glu Leu Arg Arg Asn Lys Ala His Arg Ser
    1               5                  10                  15

AAA TGC ATG CAG ATC CAG CTT TCC GCA ACT CAT CTT AGA GCT TCT TCC         94
Lys Cys Met Gln Ile Gln Leu Ser Ala Thr His Leu Arg Ala Ser Ser
                20                  25                  30

ATT CCC CAT TGG GCT TCA TTC TCT AAG ACC CCT TGG CCT TTA GGA AG         141
Ile Pro His Trp Ala Ser Phe Ser Lys Thr Pro Trp Pro Leu Gly Arg
            35                  40                  45
```

Figure 13 N

CODING SEGMENT L:

```
  Tyr Val Ser Ala Met Thr Thr Pro Ala Arg Met Ser Pro Val Asp
G TAT GTA TCA GCA ATG ACC ACC CCG GCT CGT ATG TCA CCT GTA GAT          46
| ||| ||  ||| ||  ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
G TAT GTG TCA GCC ATG ACC ACC CCG GCT CGT ATG TCA CCT GTA GAT

Phe His Thr Pro Ser Ser Pro Lys Ser Pro Pro Ser Glu Met Ser Pro
TTC CAC ACG CCA AGC TCC CCC AAG TCA CCC CCT TCG GAA ATG TCC CCG          94
||| ||| ||| ||| ||| ||| ||| ||  ||  ||| ||| ||| ||| ||| ||| ||
TTC CAC ACG CCA AGC TCC CCC AAA TCG CCC CCT TCG GAA ATG TCT CCA

Pro Val Ser Ser Thr Thr Val Ser Met Pro Ser Met Ala Val Ser Pro
CCC GTG TCC AGC ACG ACG GTC TCC ATG CCC TCC ATG GCG GTC AGT CCC         142
||| ||| ||| ||| | | ||| ||  ||| ||| ||  ||| ||| ||| ||| ||  |||
CCC GTG TCC AGC ATG ACG GTG TCC ATG CCT TCC ATG GCG GTC AGC CCC
              M

Phe Val Glu Glu Glu Arg Pro Leu Leu Leu Val Thr Pro Pro Arg Leu
TTC GTG GAA GAG GAG AGA CCC CTG CTC CTT GTG ACG CCA CCA CGG CTG         190
|||  ||  ||| ||  ||| ||| ||  ||  ||  ||  ||| ||  ||| |||  ||  |||
TTC ATG GAA GAA GAG AGA CCT CTA CTT CTC GTG ACA CCA CCA AGG CTG
    N

Arg Glu Lys  -  Tyr Asp His His Ala Gln Gln Phe Asn Ser Phe His
CGG GAG AAG ... TAT GAC CAC CAC GCC CAG CAA TTC AAC TCG TTC CAC         238
||| ||| |||     | | ||| ||  |||  |  ||| ||  ||| | |  ||  ||| |||
CGG GAG AAG AAG TTT GAC CAT CAC CCT CAG CAG TTC AGC TCC TTC CAC
          K   F               P

Cys Asn Pro Ala His Glu Ser Asn Ser Leu Pro Pro Ser Pro Leu Arg
TGC AAC CCC GCG CAT GAG AGC AAC AGC CTG CCC CCC AGC CCC TTG AGG         286
  | ||| ||| ||| ||| ||  ||  ||| ||| ||  ||   |  ||| ||| ||| |||
CAC AAC CCC GCG CAT GAC AGT AAC AGC CTC CCT GCT AGC CCC TTG AGG
N               D                       A
```

Figure 13 N'

```
Ile Val Glu Asp Glu Glu Tyr Glu Thr Thr Gln Glu Tyr Glu Pro Ala
ATA GTG GAG GAT GAG GAA TAT GAA ACG ACC CAG GAG TAC GAA CCA GCT      334
||| ||| ||| ||| ||| ||  ||| ||| ||| ||| ||  ||| ||| ||  ||| ||
ATA GTG GAG GAT GAG GAG TAT GAA ACG ACC CAA GAG TAC GAG CCA GCC

Gln Glu Pro Val Lys Lys Leu Thr Asn Ser Ser Arg Arg Ala Lys Arg
CAA GAG CCG GTT AAG AAA CTC ACC AAC AGC AGC CGG CGG GCC AAA AGA      382
||| ||| ||  ||| ||| ||| |||  ||  |||     |||  ||| ||| ||| ||| |||
CAA GAG CCT GTT AAG AAA CTC GCC AA. ..T AGC CGG CGG GCC AAA AGA
                          A

Thr Lys Pro Asn Gly His Ile Ala His Arg Leu Glu Met Asp Asn Asn
ACC AAG CCC AAT GGT CAC ATT GCC CAC AGG TTG GAA ATG GAC AAC AAC      430
||| ||| ||| ||| ||  ||| ||| ||   ||  ||  |||  ||  ||| | |  |||
ACC AAG CCC AAT GGC CAC ATT GCT AAC AGA TTG GAA GTG GAC AGC AAC
                              N             V       S

Thr Gly Ala Asp Ser Ser Asn Ser Glu Ser Glu Thr Glu Asp Glu Arg
ACA GGC GCT GAC AGC AGT AAC TCA GAG AGC GAA ACA GAG GAT GAA AGA      478
|||  ||  |   |  ||| ||| ||| ||| ||| ||  |||  |||  ||  |||  ||| |||
ACA AGC TCC CAG AGC AGT AAC TCA GAG AGT GAA ACA GAA GAT GAA AGA
    S   S   Q
```

Figure 13 N''

```
Val Gly Glu Asp Thr Pro Phe Leu Ala Ile Gln Asn Pro Leu Ala Ala
GTA GGA GAA GAT ACG CCT TTC CTG GCC ATA CAG AAC CCC CTG GCA GCC      526
||| ||  ||| ||| ||| ||| ||| ||| | | ||| ||| ||| ||| ||| ||| |||
GTA GGT GAA GAT ACG CCT TTC CTG GGC ATA CAG AAC CCC CTG GCA GCC
                               G

Ser Leu Glu Ala Ala Pro Ala Phe Arg Leu Val Asp Ser Arg Thr Asn
AGT CTC GAG GCG GCC CCT GCC TTC CGC CTG GTC GAC AGC AGG ACT AAC      574
||| ||  ||| ||  |  ||| ||| ||| ||| ||| |  ||| ||| ||| ||| |||
AGT CTT GAG GCA ACA CCT GCC TTC CGC CTG GCT GAC AGC AGG ACT AAC
    -  -       T                       A

Pro Thr Gly Gly Phe Ser Pro Gln Glu Glu Leu Gln Ala Arg Leu Ser
CCA ACA GGC GGC TTC TCT CCG CAG GAA GAA TTG CAG GCC AGG CTC TCC      622
|||  ||  ||| ||  ||| ||  |  ||| ||| ||| |  ||| ||| ||| ||  ||
CCA GCA GGC CGC TTC TCG ACA CAG GAA GAA ATC CAG GCC AGG CTG TCT
   A       R           T                 I

Gly Val Ile Ala Asn Gln Asp Pro Ile Ala Val  *
GGT GTA ATC GCT AAC CAA GAC CCT ATC GCT GTC TAA AAC CGA AAT ACA      672
 ||  ||| ||  ||| ||| ||| ||| ||| ||  ||| ||  ||| ||| | | ||| | |
AGT GTA ATT GCT AAC CAA GAC CCT ATT GCT GTA TAA AAC CTA AAT AAA
S

CCC ATA GAT TCA CCT GTA AAA CTT TAT TTT ATA TAA TAA AGT ATT CCA      718
| | ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
CAC ATA GAT TCA CCT GTA AAA CTT TAT TTT ATA TAA TAA AGT ATT CCA

                                                                    733
CCT TAA ATT AAA CAA
||| ||| ||| ||| |||
CCT TAA ATT AAA CAA
```

Figure 13 O

HUMAN CODING SEGMENT E:

```
ATG AGA TGG CGA CGC GCC CCG CGC CGC TCC GGG CGT CCC GGC CCC CGG          48
Met Arg Trp Arg Arg Ala Pro Arg Arg Ser Gly Arg Pro Gly Pro Arg
1               5                   10                  15

GCC CAG CGC CCC GGC TCC GCC GCC CGC TCG TCG CCG CCG CTG CCG CTG          96
Ala Gln Arg Pro Gly Ser Ala Ala Arg Ser Ser Pro Pro Leu Pro Leu
            20                  25                  30

CTG CCA CTA CTG CTG CTG CTG GGG ACC GCG GCC CTG GCG CCG GGG GCG         144
Leu Pro Leu Leu Leu Leu Leu Gly Thr Ala Ala Leu Ala Pro Gly Ala
        35                  40                  45

GCG GCC GGC AAC GAG GCG GCT CCC GCG GGG GCC TCG GTG TGC TAC TCG         192
Ala Ala Gly Asn Glu Ala Ala Pro Ala Gly Ala Ser Val Cys Tyr Ser
    50                  55                  60

TCC CCG CCC AGC GTG GGA TCG GTG CAG GAG CTA GCT CAG CGC GCC GCG         240
Ser Pro Pro Ser Val Gly Ser Val Gln Glu Leu Ala Gln Arg Ala Ala
65                  70                  75                  80

GTG GTG ATC GAG GGA AAG GTG CAC CCG CAG CGG CGG CAG CAG GGG GCA         288
Val Val Ile Glu Gly Lys Val His Pro Gln Arg Arg Gln Gln Gly Ala
                85                  90                  95

CTC GAC AGG AAG GCG GCG GCG GCG GCG GGC GAG GCA GGG GCG TGG GGC         336
Leu Asp Arg Lys Ala Ala Ala Ala Ala Gly Glu Ala Gly Ala Trp Gly
            100                 105                 110

GGC GAT CGC GAG CCG CCA GCC GCG GGC CCA CGG GCG CTG GGG CCG CCC         384
Gly Asp Arg Glu Pro Pro Ala Ala Gly Pro Arg Ala Leu Gly Pro Pro
        115                 120                 125

GCC GAG GAG CCG CTG CTC GCC GCC AAC GGG ACC GTG CCC TCT TGG CCC         432
Ala Glu Glu Pro Leu Leu Ala Ala Asn Gly Thr Val Pro Ser Trp Pro
    130                 135                 140

ACC GCC CCG GTG CCC AGC GCC GGC GAG CCC GGG GAG GAG GCG CCC TAT         480
Thr Ala Pro Val Pro Ser Ala Gly Glu Pro Gly Glu Glu Ala Pro Tyr
145                 150                 155                 160

CTG GTG AAG GTG CAC CAG GTG TGG GCG GTG AAA GCC GGG GGC TTG AAG         528
Leu Val Lys Val His Gln Val Trp Ala Val Lys Ala Gly Gly Leu Lys
                165                 170                 175

AAG GAC TCG CTG CTC ACC GTG CGC CTG GGG ACC TGG GGC CAC CCC GCC         576
Lys Asp Ser Leu Leu Thr Val Arg Leu Gly Thr Trp Gly His Pro Ala
            180                 185                 190

TTC CCC TCC TGC GGG AGG CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC         624
Phe Pro Ser Cys Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe
        195                 200                 205

ATG GAG CCC GAC GCC AAC AGC ACC AGC CGC GCG CCG GCC GCC TTC CGA         672
Met Glu Pro Asp Ala Asn Ser Thr Ser Arg Ala Pro Ala Ala Phe Arg
    210                 215                 220

GCC TCT TTC CCC CCT CTG GAG ACG GGC CGG AAC CTC AAG AAG GAG GTC         720
Ala Ser Phe Pro Pro Leu Glu Thr Gly Arg Asn Leu Lys Lys Glu Val
225                 230                 235                 240

AGC CGG GTG CTG TGC AAG CGG TGC G                                       745
Ser Arg Val Leu Cys Lys Arg Cys
                245
```

Figure 14 A

```
AGTTTCCCCC CCCAACTTGT CGGAACTCTG GGCTCGCGCG CAGGGCAGGA GCGGAGCGGC      60

GGCGGCTGCC CAGGCGATGC GAGCGCGGGC CGGACGGTAA TCGCCTCTCC CTCCTCGGGC     120

TGCGAGCGCG CCGGACCGAG GCAGCGACAG GAGCGGACCG CGGCGGGAAC CGAGGACTCC     180

CCAGCGGCGC GCCAGCAGGA GCCACCCCGC GAGCGTGCGA CCGGGACGGA GCGCCCGCCA     240

GTCCCAGGTG GCCCGGACCG CACGTTGCGT CCCCGCGCTC CCCGCCGGCG ACAGGAGACG     300

CTCCCCCCCA CGCCGCGCGC GCCTCGGCCC GGTCGCTGGC CCGCCTCCAC TCCGGGGACA     360

AACTTTTCCC GAAGCCGATC CCAGCCCTCG GACCCAAACT TGTCGCGCGT CGCCTTCGCC     420

GGGAGCCGTC CGCGCAGAGC GTGCACTTCT CGGGCGAG ATG TCG GAG CGC AGA          475
                                          Met Ser Glu Arg Arg
                                            1               5

GAA GGC AAA GGC AAG GGG AAG GGC GGC AAG AAG GAC CGA GGC TCC GGG        523
Glu Gly Lys Gly Lys Gly Lys Gly Gly Lys Lys Asp Arg Gly Ser Gly
                    10                  15                  20

AAG AAG CCC GTG CCC GCG GCT GGC GGC CCG AGC CCA GCC TTG CCT CCC        571
Lys Lys Pro Val Pro Ala Ala Gly Gly Pro Ser Pro Ala Leu Pro Pro
            25                  30                  35

CGC TTG AAA GAG ATG AAG ATG CAG GAG TCT GTG GCA GGT TCC AAA CTA        619
Arg Leu Lys Glu Met Lys Ser Gln Glu Ser Val Ala Gly Ser Lys Leu
        40                  45                  50

GTG CTT CGG TGC GAG ACC AGT TCT GAA TAC TCC TCT CTC AAG TTC AAG        667
Val Leu Arg Cys Glu Thr Ser Ser Glu Tyr Ser Ser Leu Lys Phe Lys
    55                  60                  65

TGG TTC AAG AAT GGG AGT GAA TTA AGC CGA AAG AAC AAA CCA CAA AAC        715
Trp Phe Lys Asn Gly Ser Glu Leu Ser Arg Lys Asn Lys Pro Gln Asn
70                  75                  80                  85

ATC AAG ATA CAG AAA AGG CCG GGG AAG TCA GAA CTT CGC ATT AGC AAA        763
Ile Lys Ile Gln Lys Arg Pro Gly Lys Ser Glu Leu Arg Ile Ser Lys
                90                  95                  100

GCG TCA CTG GCT GAT TCT GGA GAA TAT ATG TGC AAA GTG ATC AGC AAA        811
Ala Ser Leu Ala Asp Ser Gly Glu Tyr Met Cys Lys Val Ile Ser Lys
            105                 110                 115
```

Figure 14 B

```
CTA GGA AAT GAC AGT GCC TCT GCC AAC ATC ACC ATT GTG GAG TCA AAC          859
Leu Gly Asn Asp Ser Ala Ser Ala Asn Ile Thr Ile Val Glu Ser Asn
        120                 125                 130

GAG ATC ACC ACT GGC ATG CCA GCC TCA ACT GAG ACA GCG TAT GTG TCT          907
Glu Ile Thr Thr Gly Met Pro Ala Ser Thr Glu Thr Ala Tyr Val Ser
    135                 140                 145

TCA GAG TCT CCC ATT AGA ATA TCA GTA TCA ACA GAA GGA ACA AAT ACT          955
Ser Glu Ser Pro Ile Arg Ile Ser Val Ser Thr Glu Gly Thr Asn Thr
150                 155                 160                 165

TCT TCA TCC ACA TCC ACA TCT ACA GCT GGG ACA AGC CAT CTT GTC AAG          1003
Ser Ser Ser Thr Ser Thr Ser Thr Ala Gly Thr Ser His Leu Val Lys
                170                 175                 180

TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGC GAG TGC TTC          1051
Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn Gly Gly Glu Cys Phe
            185                 190                 195

ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC TTG TGC AAG TGC CCA          1099
Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr Leu Cys Lys Cys Pro
        200                 205                 210

AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC          1147
Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe
    215                 220                 225

TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT GAA TAGGCGCATG               1193
Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro Glu
230                 235                 240

CTCAGTCGGT GCCGCTTTCT TGTTGCCGCA TCTCCCCTCA GATTCAACCT AGAGCTAGAT       1253

GCGTTTTACC AGGTCTAACA TTGACTGCCT CTGCCTGTCG CATGAGAACA TTAACACAAG       1313

CGATTGTATG ACTTCCTCTG TCCGTGACTA GTGGGCTCTG AGCTACTCGT AGGTGCGTAA       1373

GGCTCCAGTG TTTCTGAAAT TGATCTTGAA TTACTGTGAT ACGACATGAT AGTCCCTCTC       1433

ACCCAGTGCA ATGACAATAA AGGCCTTGAA AAGTCTCACT TTTATTGAGA AAATAAAAAT       1493

CGTTCCACGG GACAGTCCCT CTTCTTTATA AAATGACCCT ATCCTTGAAA AGGAGGTGTG       1553

TTAAGTTGTA ACCAGTACAC ACTTGAAATG ATGGTAAGTT CGCTTCGGTT CAGAATGTGT       1613

TCTTTCTGAC AAATAAACAG AATAAAAAAA AAAAAAAAAA A                           1654
```

Figure 15 A

```
CAT CAN GTG TGG GCG GCG AAA GCC GGG GGC TTG AAG AAG GAC TCG CTG                48
His Gln Val Trp Ala Ala Lys Ala Gly Gly Leu Lys Lys Asp Ser Leu
1               5                   10                  15

CTC ACC GTG CGC CTG GGC GCC TGG GGC CAC CCC GCC TTC CCC TCC TGC                96
Leu Thr Val Arg Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser Cys
            20                  25                  30

GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC GAG               144
Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro Glu
        35                  40                  45

GCC AAC AGC AGC GGC GGG CCC GGC CGC CTT CCG AGC CTC CTT CCC CCC               192
Ala Asn Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro Pro
    50                  55                  60

TCT CGA GAC GGG CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT GTG               240
Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Gln Pro Gly Ala Val
65                  70                  75                  80

CAA CGG TGC GCC TTG CCT CCC CGC TTG AAA GAG ATG AAG AGT CAG GAG               288
Gln Arg Cys Ala Leu Pro Pro Arg Leu Lys Glu Met Lys Ser Gln Glu
                85                  90                  95

TCT GTG GCA GGT TCC AAA CTA GTG CTT CGG TGC GAG ACC AGT TCT GAA               336
Ser Val Ala Gly Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu
            100                 105                 110

TAC TCC TCT CTC AAG TTC AAG TGG TTC AAG AAT GGG AGT GAA TTA AGC               384
Tyr Ser Ser Leu Lys Phe Lys Trp Phe Lys Asn Gly Ser Glu Leu Ser
        115                 120                 125

CGA AAG AAC AAA CCA GAA AAC ATC AAG ATA CAG AAA AGG CCG GGG AAG               432
Arg Lys Asn Lys Pro Glu Asn Ile Lys Ile Gln Lys Arg Pro Gly Lys
    130                 135                 140

TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA TAT               480
Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr
145                 150                 155                 160

ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC               528
Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn
                165                 170                 175
```

Figure 15 B

```
ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG ACA          576
Ile Thr Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly Thr
            180                 185                 190

AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT          624
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
        195                 200                 205

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC          672
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
    210                 215                 220

TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT          720
Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn
225                 230                 235                 240

GTG CCC ATG AAA GTC CAA ACC CAA GAA AAG TGC CCA AAT GAG TTT ACT          768
Val Pro Met Lys Val Gln Thr Gln Glu Lys Cys Pro Asn Glu Phe Thr
                245                 250                 255

GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC TAC AGT ACG TCC          816
Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe Tyr Ser Thr Ser
            260                 265                 270

ACT CCC TTT CTG TCT CTG CCT GAA TAGCGCATCT CAGTCGGTGC CGCTTTCTTG         870
Thr Pro Phe Leu Ser Leu Pro Glu
        275                 280

TTGCCGCATC TCCCCTCAGA TTCCNCCTAG AGCTAGATGC GTTTTACCAG GTCTAACATT        930

GACTGCCTCT GCCTGTCGCA TGAGAACATT AACACAAGCG ATTGTATGAC TTCCTCTGTC        990

CGTGACTAGT GGGCTCTGAG CTACTCGTAG GTGCGTAAGG CTCCAGTGTT TCTGAAATTG       1050

ATCTTGAATT ACTGTGATAC GACATGATAG TCCCTCTCAC CCAGTGCAAT GACAATAAAG       1110

GCCTTGAAAA GTCAAAAAAA AAAAAAAAAA                                        1140
```

Figure 16 A

```
G AAG TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA      49
  Lys Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu
   1               5                  10                  15

TAT ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC      97
Tyr Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala
            20                  25                  30

AAC ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG     145
Asn Ile Thr Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly
        35                  40                  45

ACA AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG     193
Thr Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val
    50                  55                  60

AAT GGA GGC GAC TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA     241
Asn Gly Gly Asp Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg
65                  70                  75                  80

TAC TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG     289
Tyr Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu
                85                  90                  95

AAT GTG CCC ATG AAA GTC CAA ACC CAA GAA AAA GCG GAG GAG CTC TAC     337
Asn Val Pro Met Lys Val Gln Thr Gln Glu Lys Ala Glu Glu Leu Tyr
            100                 105                 110

CAG AAG AGA GTG CTC ACC ATT ACC GGC ATT TGC ATC GCG CTG CTC GTG     385
Gln Lys Arg Val Leu Thr Ile Thr Gly Ile Cys Ile Ala Leu Leu Val
        115                 120                 125

GTT GGC ATC ATG TGT GTG GTG GTC TAC TGC AAA ACC AAG AAA CAA CGG     433
Val Gly Ile Met Cys Val Val Val Tyr Cys Lys Thr Lys Lys Gln Arg
    130                 135                 140

AAA AAG CTT CAT GAC CGG CTT CGG CAG AGC CTT CGG TCT GAA AGA AAC     481
Lys Lys Leu His Asp Arg Leu Arg Gln Ser Leu Arg Ser Glu Arg Asn
145                 150                 155                 160

ACC ATG ATG AAC GTA GCC AAC GGG CCC CAC CAC CCC AAT CCG CCC CCC     529
Thr Met Met Asn Val Ala Asn Gly Pro His His Pro Asn Pro Pro Pro
                165                 170                 175

GAG AAC GTG CAG CTG GTG AAT CAA TAC GTA TCT AAA AAT GTC ATC TCT     577
Glu Asn Val Gln Leu Val Asn Gln Tyr Val Ser Lys Asn Val Ile Ser
            180                 185                 190
```

Figure 16 B

```
AGC GAG CAT ATT GTT GAG AGA GAG GCG GAG AGC TCT TTT TCC ACC AGT          625
Ser Glu His Ile Val Glu Arg Glu Ala Glu Ser Ser Phe Ser Thr Ser
        195                 200                 205

CAC TAC ACT TCG ACA GCT CAT CAT TCC ACT ACT GTC ACT CAG ACT CCC          673
His Tyr Thr Ser Thr Ala His His Ser Thr Thr Val Thr Gln Thr Pro
    210                 215                 220

AGT CAC AGC TGG AGC AAT GGA CAC ACT GAA AGC ATC ATT TCG GAA AGC          721
Ser His Ser Trp Ser Asn Gly His Thr Glu Ser Ile Ile Ser Glu Ser
225                 230                 235                 240

CAC TCT GTC ATC GTG ATG TCA TCC GTA GAA AAC AGT AGG CAC AGC AGC          769
His Ser Val Ile Val Met Ser Ser Val Glu Asn Ser Arg His Ser Ser
                245                 250                 255

CCG ACT GGG GGC CCG AGA GGA CGT CTC AAT GGC TTG GGA GGC CCT CGT          817
Pro Thr Gly Gly Pro Arg Gly Arg Leu Asn Gly Leu Gly Gly Pro Arg
            260                 265                 270

GAA TGT AAC AGC TTC CTC AGG CAT GCC AGA GAA ACC CCT GAC TCC TAC          865
Glu Cys Asn Ser Phe Leu Arg His Ala Arg Glu Thr Pro Asp Ser Tyr
        275                 280                 285

CGA GAC TCT CCT CAT AGT GAA AGA CAT AAC CTT ATA GCT GAG CTA AGG          913
Arg Asp Ser Pro His Ser Glu Arg His Asn Leu Ile Ala Glu Leu Arg
    290                 295                 300

AGA AAC AAG GCC CAC AGA TCC AAA TGC ATG CAG ATC CAG CTT TCC GCA          961
Arg Asn Lys Ala His Arg Ser Lys Cys Met Gln Ile Gln Leu Ser Ala
305                 310                 315                 320

ACT CAT CTT AGA GCT TCT TCC ATT CCC CAT TGG GCT TCA TTC TCT AAG         1009
Thr His Leu Arg Ala Ser Ser Ile Pro His Trp Ala Ser Phe Ser Lys
                325                 330                 335

ACC CCT TGG CCT TTA GGA AGG TAT GTA TCA GCA ATG ACC ACC CCG GCT         1057
Thr Pro Trp Pro Leu Gly Arg Tyr Val Ser Ala Met Thr Thr Pro Ala
            340                 345                 350

CGT ATG TCA CCT GTA GAT TTC CAC ACG CCA AGC TCC CCC AAG TCA CCC         1105
Arg Met Ser Pro Val Asp Phe His Thr Pro Ser Ser Pro Lys Ser Pro
        355                 360                 365

CCT TCG GAA ATG TCC CCG CCC GTG TCC AGC ACG ACG GTC TCC ATG CCC         1153
Pro Ser Glu Met Ser Pro Pro Val Ser Ser Thr Thr Val Ser Met Pro
    370                 375                 380
```

Figure 16 C

```
TCC ATG GCG GTC AGT CCC TTC GTG GAA GAG GAG AGA CCC CTG CTC CTT          1201
Ser Met Ala Val Ser Pro Phe Val Glu Glu Glu Arg Pro Leu Leu Leu
385                 390                 395                 400

GTG ACG CCA CCA CGG CTG CGG GAG AAG TAT GAC CAC CAC GCC CAG CAA          1249
Val Thr Pro Pro Arg Leu Arg Glu Lys Tyr Asp His His Ala Gln Gln
                405                 410                 415

TTC AAC TCG TTC CAC TGC AAC CCC GCG CAT GAG AGC AAC AGC CTG CCC          1297
Phe Asn Ser Phe His Cys Asn Pro Ala His Glu Ser Asn Ser Leu Pro
            420                 425                 430

CCC AGC CCC TTG AGG ATA GTG GAG GAT GAG GAA TAT GAA ACG ACC CAG          1345
Pro Ser Pro Leu Arg Ile Val Glu Asp Glu Glu Tyr Glu Thr Thr Gln
        435                 440                 445

GAG TAC GAA CCA GCT CAA GAG CCG GTT AAG AAA CTC ACC AAC AGC AGC          1393
Glu Tyr Glu Pro Ala Gln Glu Pro Val Lys Lys Leu Thr Asn Ser Ser
    450                 455                 460

CGG CGG GCC AAA AGA ACC AAG CCC AAT GGT CAC ATT GCC CAC AGG TTG          1441
Arg Arg Ala Lys Arg Thr Lys Pro Asn Gly His Ile Ala His Arg Leu
465                 470                 475                 480

GAA ATG GAC AAC AAC ACA GGC GCT GAC AGC AGT AAC TCA GAG AGC GAA          1489
Glu Met Asp Asn Asn Thr Gly Ala Asp Ser Ser Asn Ser Glu Ser Glu
                485                 490                 495

ACA GAG GAT GAA AGA GTA GGA GAA GAT ACG CCT TTC CTG GCC ATA CAG          1537
Thr Glu Asp Glu Arg Val Gly Glu Asp Thr Pro Phe Leu Ala Ile Gln
            500                 505                 510

AAC CCC CTG GCA GCC AGT CTC GAG GCG GCC CCT GCC TTC CGC CTG GTC          1585
Asn Pro Leu Ala Ala Ser Leu Glu Ala Ala Pro Ala Phe Arg Leu Val
        515                 520                 525

GAC AGC AGG ACT AAC CCA ACA GGC GGC TTC TCT CCG CAG GAA GAA TTG          1633
Asp Ser Arg Thr Asn Pro Thr Gly Gly Phe Ser Pro Gln Glu Glu Leu
    530                 535                 540

CAG GCC AGG CTC TCC GGT GTA ATC GCT AAC CAA GAC CCT ATC GCT GTC          1681
Gln Ala Arg Leu Ser Gly Val Ile Ala Asn Gln Asp Pro Ile Ala Val
545                 550                 555                 560

TAAAACCGAA ATACACCCAT AGATTCACCT GTAAAACTTT ATTTTATATA ATAAAGTATT        1741

CCACCTTAAA TTAAACAAAA AAA                                               1764
```

Figure 17 A

F-B-A'	F-E-B-A'
F-B-A-C-C/D-D	F-E-B-A-C-C/D-D
F-B-A-C-C/D-H	F-E-B-A-C-C/D-H
F-B-A-C-C/D-H-L	F-E-B-A-C-C/D-H-L
F-B-A-C-C/D-H-K-L	F-E-B-A-C-C/D-H-K-L
F-B-A-C-C/D-D'-H	F-E-B-A-C-C/D-D'-H
F-B-A-C-C/D-D'-H-L	F-E-B-A-C-C/D-D'-H-L
F-B-A-C-C/D-D'-H-K-L	F-E-B-A-C-C/D-D'-H-K-L
F-B-A-C-C/D'-D	F-E-B-A-C-C/D'-D
F-B-A-C-C/D'-H	F-E-B-A-C-C/D'-H
F-B-A-C-C/D'-H-L	F-E-B-A-C-C/D'-H-L
F-B-A-C-C/D'-H-K-L	F-E-B-A-C-C/D'-H-K-L
F-B-A-C-C/D'-D'-H	F-E-B-A-C-C/D'-D'-H
F-B-A-C-C/D'-D'-H-L	F-E-B-A-C-C/D'-D'-H-L
F-B-A-C-C/D'-D'-'H-K-L	F-E-B-A-C-C/D'-D'-'H-K-L
F-B-A-C-C/D-C/D'-D	F-E-B-A-C-C/D-C/D'-D
F-B-A-C-C/D-C/D'-H	F-E-B-A-C-C/D-C/D'-H
F-B-A-C-C/D-C/D'-H-L	F-E-B-A-C-C/D-C/D'-H-L
F-B-A-C-C/D-C/D'-H-K-L	F-E-B-A-C-C/D-C/D'-H-K-L
F-B-A-C-C/D-C/D'-D'-H	F-E-B-A-C-C/D-C/D'-D'-H
F-B-A-C-C/D-C/D'-D'-H-L	F-E-B-A-C-C/D-C/D'-D'-H-L
F-B-A-C-C/D-C/D'-D'-H-K-L	F-E-B-A-C-C/D-C/D'-D'-H-K-L
F-B-A-G-C-C/D-D	F-E-B-A-G-C-C/D-D
F-B-A-G-C-C/D-H	F-E-B-A-G-C-C/D-H
F-B-A-G-C-C/D-H-L	F-E-B-A-G-C-C/D-H-L
F-B-A-G-C-C/D-H-K-L	F-E-B-A-G-C-C/D-H-K-L
F-B-A-G-C-C/D-D'-H	F-E-B-A-G-C-C/D-D'-H
F-B-A-G-C-C/D-D'-H-L	F-E-B-A-G-C-C/D-D'-H-L
F-B-A-G-C-C/D-D'-H-K-L	F-E-B-A-G-C-C/D-D'-H-K-L
F-B-A-G-C-C/D'-D	F-E-B-A-G-C-C/D'-D
F-B-A-G-C-C/D'-H	F-E-B-A-G-C-C/D'-H
F-B-A-G-C-C/D'-H-L	F-E-B-A-G-C-C/D'-H-L
F-B-A-G-C-C/D'-H-K-L	F-E-B-A-G-C-C/D'-H-K-L
F-B-A-G-C-C/D'-D'-H	F-E-B-A-G-C-C/D'-D'-H
F-B-A-G-C-C/D'-D'-H-L	F-E-B-A-G-C-C/D'-D'-H-L
F-B-A-G-C-C/D'-D'-'H-K-L	F-E-B-A-G-C-C/D'-D'-'H-K-L
F-B-A-G-C-C/D-C/D'-D	F-E-B-A-G-C-C/D-C/D'-D
F-B-A-G-C-C/D-C/D'-H	F-E-B-A-G-C-C/D-C/D'-H
F-B-A-G-C-C/D-C/D'-H-L	F-E-B-A-G-C-C/D-C/D'-H-L
F-B-A-G-C-C/D-C/D'-H-K-L	F-E-B-A-G-C-C/D-C/D'-H-K-L
F-B-A-G-C-C/D-C/D'-D'-H	F-E-B-A-G-C-C/D-C/D'-D'-H
F-B-A-G-C-C/D-C/D'-D'-H-L	F-E-B-A-G-C-C/D-C/D'-D'-H-L
F-B-A-G-C-C/D-C/D'-D'-H-K-L	F-E-B-A-G-C-C/D-C/D'-D'-H-K-L

Figure 17 B

E-B-A'

E-B-A-C-C/D-D
E-B-A-C-C/D-H
E-B-A-C-C/D-H-L
E-B-A-C-C/D-H-K-L
E-B-A-C-C/D-D'-H
E-B-A-C-C/D-D'-H-L
E-B-A-C-C/D-D'-H-K-L
E-B-A-C-C/D'-D
E-B-A-C-C/D'-H
E-B-A-C-C/D'-H-L
E-B-A-C-C/D'-H-K-L
E-B-A-C-C/D'-D'-H
E-B-A-C-C/D'-D'-H-L
E-B-A-C-C/D'-D'-H-K-L
E-B-A-C-C/D-C/D'-D
E-B-A-C-C/D-C/D'-H
E-B-A-C-C/D-C/D'-H-L
E-B-A-C-C/D-C/D'-H-K-L
E-B-A-C-C/D-C/D'-D'-H
E-B-A-C-C/D-C/D'-D'-H-L
E-B-A-C-C/D-C/D'-D'-H-K-L

E-B-A-G-C-C/D-D
E-B-A-G-C-C/D-H
E-B-A-G-C-C/D-H-L
E-B-A-G-C-C/D-H-K-L
E-B-A-G-C-C/D-D'-H
E-B-A-G-C-C/D-D'-H-L
E-B-A-G-C-C/D-D'-H-K-L
E-B-A-G-C-C/D'-D
E-B-A-G-C-C/D'-H
E-B-A-G-C-C/D'-H-L
E-B-A-G-C-C/D'-H-K-L
E-B-A-G-C-C/D'-D'-H
E-B-A-G-C-C/D'-D'-H-L
E-B-A-G-C-C/D'-D'-H-K-L
E-B-A-G-C-C/D-C/D'-D
E-B-A-G-C-C/D-C/D'-H
E-B-A-G-C-C/D-C/D'-H-L
E-B-A-G-C-C/D-C/D'-H-K-L
E-B-A-G-C-C/D-C/D'-D'-H
E-B-A-G-C-C/D-C/D'-D'-H-L
E-B-A-G-C-C/D-C/D'-D'-H-K-L

Figure 18

```
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT                48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1               5                   10                  15

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC                96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
            20                  25                  30

TTG TGC AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC               144
Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr
        35                  40                  45

GTA ATG GCC AGC TTC TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT               192
Val Met Ala Ser Phe Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro
    50                  55                  60

GAA TAG                                                                      198
Glu
65
```

Figure 19

```
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT          48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1               5                  10                  15

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC          96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
            20                  25                  30

TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT         144
Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn
        35                  40                  45

GTG CCC ATG AAA GTC CAA ACC CAA GAA AAA GCG GAG GAG CTC TAC TAA         192
Val Pro Met Lys Val Gln Thr Gln Glu Lys Ala Glu Glu Leu Tyr
    50                  55                  60
```

Figure 20

```
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT                48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1               5                   10                  15

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC                96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
            20                  25                  30

TTG TGC AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC               144
Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr
        35                  40                  45

GTA ATG GCC AGC TTC TAC AAA GCG GAG GAG CTC TAC TAA                           183
Val Met Ala Ser Phe Tyr Lys Ala Glu Glu Leu Tyr
    50                  55                  60
```

Figure 21

```
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT          48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1               5                   10                  15

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC          96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
            20                  25                  30

TTG TGC AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC         144
Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr
        35                  40                  45

GTA ATG GCC AGC TTC TAC AAG CAT CTT GGG ATT GAA TTT ATG GAG AAA         192
Val Met Ala Ser Phe Tyr Lys His Leu Gly Ile Glu Phe Met Glu Lys
    50                  55                  60

GCG GAG GAG CTC TAC TAA                                             210
Ala Glu Glu Leu Tyr
65
```

Figure 22

```
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT                48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1               5                   10                  15

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC                96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
            20                  25                  30

TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT               144
Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn
        35                  40                  45

GTG CCC ATG AAA GTC CAA ACC CAA GAA AAG TGC CCA AAT GAG TTT ACT               192
Val Pro Met Lys Val Gln Thr Gln Glu Lys Cys Pro Asn Glu Phe Thr
    50                  55                  60

GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC TAC AGT ACG TCC               240
Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe Tyr Ser Thr Ser
65                  70                  75                  80

ACT CCC TTT CTG TCT CTG CCT GAA TAG                                           267
Thr Pro Phe Leu Ser Leu Pro Glu
                85
```

Figure 23

```
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT      48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1               5                  10                  15

GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC      96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
            20                  25                  30

TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT     144
Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn
        35                  40                  45

GTG CCC ATG AAA GTC CAA ACC CAA GAA AAG TGC CCA AAT GAG TTT ACT     192
Val Pro Met Lys Val Gln Thr Gln Glu Lys Cys Pro Asn Glu Phe Thr
    50                  55                  60

GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC TAC AAA GCG GAG     240
Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe Tyr Lys Ala Glu
65                  70                  75                  80

GAG CTC TAC TAA                                                     252
Glu Leu Tyr
```

Figure 24 A

```
GGAATTCCTT TTTTTTTTTT TTTTTTTCTT NNTTTTTTTT TGCCCTTATA CCTCTTCGCC          60

TTTCTGTGGT TCCATCCACT TCTTCCCCCT CCTCCTCCCA TAAACAACTC TCCTACCCCT         120

GCACCCCCAA TAAATAAATA AAAGGAGGAG GGCAAGGGGG GAGGAGGAGG AGTGGTGCTG         180

CGAGGGGAAG GAAAAGGGAG GCAGCGCGAG AAGAGCCGGG CAGAGTCCGA ACCGACAGCC         240

AGAAGCCCGC ACGCACCTCG CACC ATG AGA TGG CGA CGC GCC CCG CGC CGC              291
                           Met Arg Trp Arg Arg Ala Pro Arg Arg
                            1               5

TCC GGG CGT CCC GGC CCC CGG GCC CAG CGC CCC GGC TCC GCC GCC CGC            339
Ser Gly Arg Pro Gly Pro Arg Ala Gln Arg Pro Gly Ser Ala Ala Arg
 10                  15                  20                  25

TCG TCG CCG CCG CTG CCG CTG CTG CCA CTA CTG CTG CTG CTG GGG ACC            387
Ser Ser Pro Pro Leu Pro Leu Leu Pro Leu Leu Leu Leu Leu Gly Thr
                                        Val Cys Leu Leu Thr Val
                                                    GGF II 09
                30                  35                  40

GCG GCC CTG GCG CCG GGG GCG GCG GCC GGC AAC GAG GCG GCT CCC GCG            435
Ala Ala Leu Ala Pro Gly Ala Ala Ala Gly Asn Glu Ala Ala Pro Ala
Ala Ala Leu Pro Pro
            45                  50                  55

GGG GCC TCG GTG TGC TAC TCG TCC CCG CCC AGC GTG GGA TCG GTG CAG            483
Gly Ala Ser Val Cys Tyr Ser Ser Pro Pro Ser Val Gly Ser Val Gln
                    Ala Ser Pro Val Ser Val Gly Ser Val Gln
                                                GGF II 08
        60                  65                  70

GAG CTA GCT CAG CGC | GCC GCG GTG GTG ATC GAG GGA AAG GTG CAC CCG          531
Glu Leu Ala Gln Arg | Ala Ala Val Val Ile Glu Gly Lys Val His Pro
Glu Leu Val Gln Arg | Trp Phe Val Val Ile Glu Gly Lys
                                GGF II 04
    75                  80                  85
```

Figure 24 B

```
CAG CGG CGG CAG CAG GGG GCA CTC GAC AGG AAG GCG GCG GCG GCG GCG          579
Gln Arg Arg Gln Gln Gly Ala Leu Asp Arg Lys Ala Ala Ala Ala Ala
 90                  95                  100                 105

GGC GAG GCA GGG GCG TGG GGC GGC GAT CGC GAG CCG CCA GCC GCG GGC          627
Gly Glu Ala Gly Ala Trp Gly Gly Asp Arg Glu Pro Pro Ala Ala Gly
                110                 115                 120

CCA CGG GCG CTG GGG CCG CCC GCC GAG GAG CCG CTG CTC GCC GCC AAC          675
Pro Arg Ala Leu Gly Pro Pro Ala Glu Glu Pro Leu Leu Ala Ala Asn
            125                 130                 135

GGG ACC GTG CCC TCT TGG CCC ACC GCC CCG GTG CCC AGC GCC GGC GAG          723
Gly Thr Val Pro Ser Trp Pro Thr Ala Pro Val Pro Ser Ala Gly Glu
        140                 145                 150

CCC GGG GAG GAG GCG CCC TAT CTG GTG AAG GTG CAC CAG GTG TGG GCG          771
Pro Gly Glu Glu Ala Pro Tyr Leu Val Lys Val His Gln Val Trp Ala
                                    Lys Val His Glu Val Trp Ala
                                        GGF II 01 & GGF II 11
    155                 160                 165

GTG AAA GCC GGG GGC TTG AAG AAG GAC TCG CTG CTC ACC GTG CGC CTG          819
Val Lys Ala Gly Gly Leu Lys Lys Asp Ser Leu Leu Thr Val Arg Leu
Ala Lys                         Asp Leu Leu Leu Xaa Val     Leu
                                    GGF II 10
170                 175                 180                 185

GGG ACC TGG GGC CAC CCC GCC TTC CCC TCC TGC GGG AGG CTC AAG GAG          867
Gly Thr Trp Gly His Pro Ala Phe Pro Ser Cys Gly Arg Leu Lys Glu
Gly Ala Trp Gly Pro Pro Ala Phe Pro Val Xaa Tyr
               GGF II 03
                190                 195                 200

GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC GAC GCC AAC AGC ACC AGC          915
Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro Asp Ala Asn Ser Thr Ser
            Tyr Ile Phe Phe Met Glu Pro Asp Ala Xaa Ser Ser Gly
                                    GGF II 02
            205                 210                 215
```

Figure 24 C

```
CGC GCG CCG GCC GCC TTC CGA GCC TCT TTC CCC CCT CTG GAG ACG GGC          963
Arg Ala Pro Ala Ala Phe Arg Ala Ser Phe Pro Pro Leu Glu Thr Gly
        220                 225                 230

CGG AAC CTC AAG AAG GAG GTC AGC CGG GTG CTG TGC AAG CGG TGC GCC         1011
Arg Asn Leu Lys Lys Glu Val Ser Arg Val Leu Cys Lys Arg Cys Ala
    235                 240                 245

TTG CCT CCC CAA TTG AAA GAG ATG AAA AGC CAG GAA TCG GCT GCA GGT         1059
Leu Pro Pro Gln Leu Lys Glu Met Lys Ser Gln Glu Ser Ala Ala Gly
250                 255                 260                 265

TCC AAA CTA GTC CTT CGG TGT GAA ACC AGT TCT GAA TAC TCC TCT CTC         1107
Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu Tyr Ser Ser Leu
        Leu Val Leu Arg
            GGF II 06
                270                 175                 180

AGA TTC AAG TGG TTC AAG AAT GGG AAT GAA TTG AAT CGA AAA AAC AAA         1155
Arg Phe Lys Trp Phe Lys Asn Gly Asn Glu Leu Asn Arg Lys Asn Lys
            185                 190                 195

CCA CAA AAT ATC AAG ATA CAA AAA AAG CCA GGG AAG TCA GAA CTT CGC         1203
Pro Gln Asn Ile Lys Ile Gln Lys Lys Pro Gly Lys Ser Glu Leu Arg
        200                 205                 210

ATT AAC AAA GCA TCA CTG GCT GAT TCT GGA GAG TAT ATG TGC AAA GTG         1251
Ile Asn Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr Met Cys Lys Val
        Lys Ala Ser Leu Ala Asp Ser Gly Glu Tyr Met Xaa Lys
                                GGF II 12
    215                 220                 225

ATC AGC AAA TTA GGA AAT GAC AGT GCC TCT GCC AAT ATC ACC ATC GTG         1299
Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn Ile Thr Ile Val
230                 235                 240                 245

GAA TCA AAC GCT ACA TCT ACA TCC ACC ACT GGG ACA AGC CAT CTT GTA         1347
Glu Ser Asn Ala Thr Ser Thr Ser Thr Thr Gly Thr Ser His Leu Val
                250                 255                 260
```

Figure 24 D

```
AAA TGT GCG GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGG GAG TGC          1395
Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn Gly Gly Glu Cys
            265                 270                 275

TTC ATG GTG AAA GAC CTT TCA AAC CCC TCG AGA TAC TTG TGC AAG TGC          1443
Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr Leu Cys Lys Cys
        280                 285                 290

CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC          1491
Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser
    295                 300                 305

TTC TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT GAA                      1530
Phe Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro Glu
400                 405                 410

TAGGAGCATG CTCAGTTGGT GCTGCTTTCT TGTTGCTGCA TCTCCCCTCA GATTCCACCT        1590

AGAGCTAGAT GTGTCTTACC AGATCTAATA TTGACTGCCT CTGCCTGTCG CATGAGAACA        1650

TTAACAAAAG CAATTGTATT ACTTCCTCTG TTCGCGACTA GTTGGCTCTG AGATACTAAT        1710

AGGTGTGTGA GGCTCCGGAT GTTTCTGGAA TTGATATTGA ATGATGTGAT ACAAATTGAT        1770

AGTCAATATC AAGCAGTGAA ATATGATAAT AAAGGCATTT CAAAGTCTCA CTTTTATTGA        1830

TAAAATAAAA ATCATTCTAC TGAACAGTCC ATCTTCTTTA TACAATGACC ACATCCTGAA        1890

AAGGGTGTTG CTAAGCTGTA ACCGATATGC ACTTGAAATG ATGGTAAGTT AATTTTGATT        1950

CAGAATGTGT TATTTGTCAC AAATAAACAT AATAAAAGGA AAAAAAAAAA AAA               2003
```

Figure 25

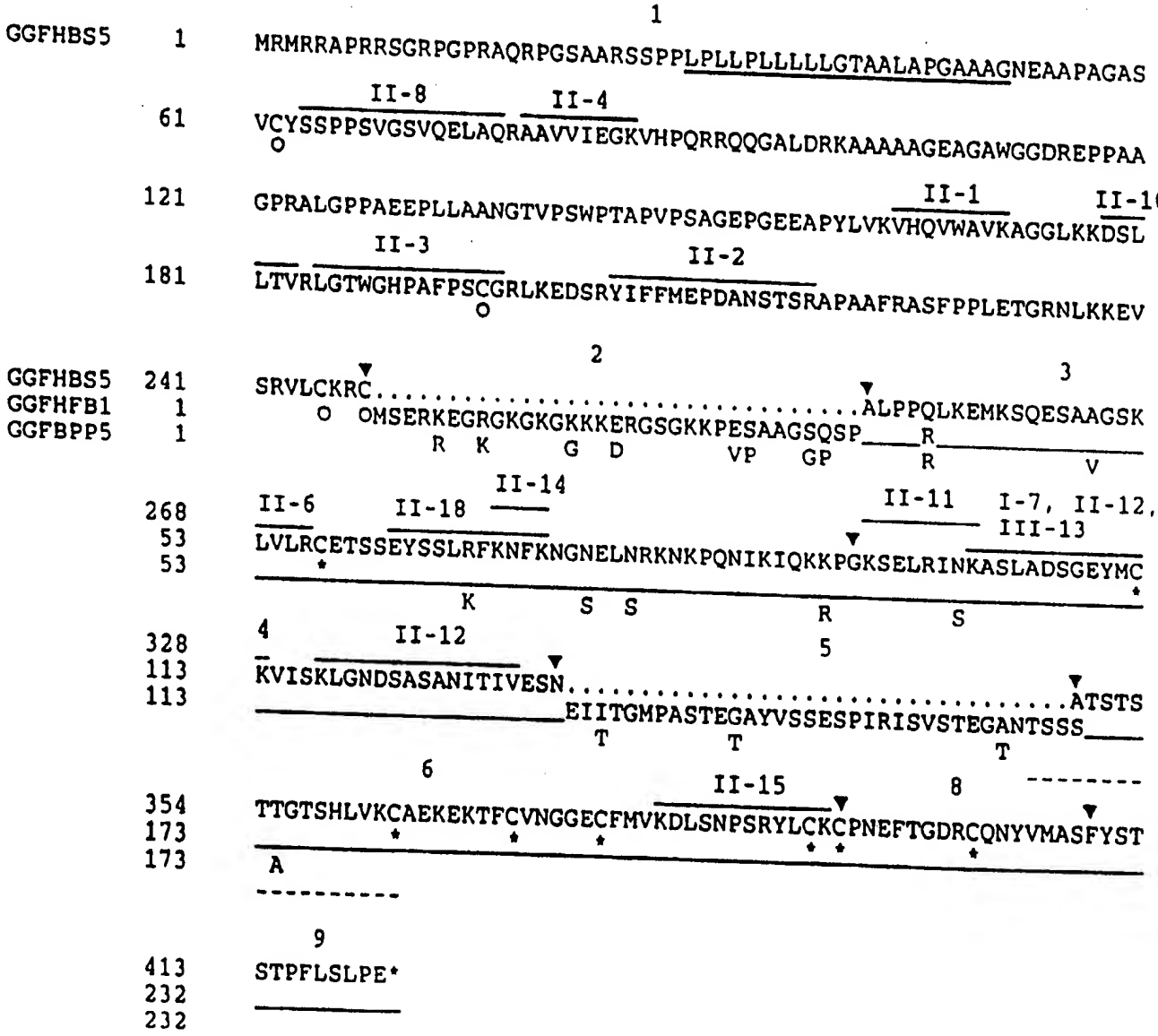

Figure 26

```
Met Arg Trp Arg Arg Ala Pro Arg Arg Ser Gly Arg Pro Gly Pro Arg
 1               5                  10                  15

Ala Gln Arg Pro Gly Ser Ala Ala Arg Ser Ser Pro Pro Leu Pro Leu
            20                  25                  30

Leu Pro Leu Leu Leu Leu Leu Gly Thr Ala Ala Leu Ala Pro Gly Ala
        35                  40                  45

Ala Ala Gly Asn Glu Ala Ala Pro Ala Gly Ala Ser Val Cys Tyr Ser
    50                  55                  60

Ser Pro Pro Ser Val Gly Ser Val Gln Glu Leu Ala Gln Arg Ala Ala
65                  70                  75                  80

Val Val Ile Glu Gly Lys Val His Pro Gln Arg Arg Gln Gln Gly Ala
                85                  90                  95

Leu Asp Arg Lys Ala Ala Ala Ala Ala Gly Glu Ala Gly Ala Trp Gly
            100                 105                 110

Gly Asp Arg Glu Pro Pro Ala Ala Gly Pro Arg Ala Leu Gly Pro Pro
        115                 120                 125

Ala Glu Glu Pro Leu Leu Ala Ala Asn Gly Thr Val Pro Ser Trp Pro
    130                 135                 140

Thr Ala Pro Val Pro Ser Ala Gly Glu Pro Gly Glu Glu Ala Pro Tyr
145                 150                 155                 160

Leu Val Lys Val His Gln Val Trp Ala Val Lys Ala Gly Gly Leu Lys
                165                 170                 175

Lys Asp Ser Leu Leu Thr Val Arg Leu Gly Thr Trp Gly His Pro Ala
            180                 185                 190

Phe Pro Ser Cys Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe
        195                 200                 205

Met Glu Pro Asp Ala Asn Ser Thr Ser Arg Ala Pro Ala Ala Phe Arg
    210                 215                 220

Ala Ser Phe Pro Pro Leu Glu Thr Gly Arg Asn Leu Lys Lys Glu Val
225                 230                 235                 240

Ser Arg Val Leu Cys Lys Arg Cys Ala Leu Pro Pro Gln Leu Lys Glu
                245                 250                 255

Met Lys Ser Gln Glu Ser Ala Ala Gly Ser Lys Leu Val Leu Arg Cys
            260                 265                 270

Glu Thr Ser Ser Glu Tyr Ser Ser Leu Arg Phe Lys Trp Phe Lys Asn
        275                 280                 285

Gly Asn Glu Leu Asn Arg Lys Asn Lys Pro Gln Asn Ile Lys Ile Gln
    290                 295                 300

Lys Lys Pro Gly Lys Ser Glu Leu Arg Ile Asn Lys Ala Ser Leu Ala
305                 310                 315                 320

Asp Ser Gly Glu Tyr Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp
                325                 330                 335
```

Figure 26 (cont.)

```
Ser Ala Ser Ala Asn Ile Thr Ile Val Glu Ser Asn Ala Thr Ser Thr
            340                 345                 350

Ser Thr Thr Gly Thr Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys
        355                 360                 365

Thr Phe Cys Val Asn Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser
    370                 375                 380

Asn Pro Ser Arg Tyr Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp
385                 390                 395                 400

Arg Cys Gln Asn Tyr Val Met Ala Ser Phe Tyr Ser Thr Ser Thr Pro
                405                 410                 415

Phe Leu Ser Leu Pro Glu *
            420
```

Figure 27

```
TCTAA AAC TAC AGA GAC TGT ATT TTC ATG ATC ATC ATA GTT CTG TGA AAT ATA      53
      Asn Tyr Arg Asp Cys Ile Phe Met Ile Ile Ile Val Leu Xaa Asn Ile
        1               5                  10                  15

CTT AAA CCG CTT TGG TCC TGA TCT TGT AGG AAG TCA GAA CTT CGC ATT          101
Leu Lys Pro Leu Trp Ser Xaa Ser Cys Arg Lys Ser Glu Leu Arg Ile
            20                  25                  30

AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA TAT ATG TGC AAA GTG ATC          149
Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu Ser Met Cys Lys Val Ile
        35                  40                  45

AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC ATC ACC ATT GTG GAG          197
Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn Ile Arg Ile Val Glu
    50                  55                  60

TCA AAC GGT AAG AGA TGC CTA CTG CGT GCT ATT TCT CAG TCT CTA AGA          245
Ser Asn Gly Lys Arg Cys Leu Leu Arg Ala Ile Ser Gln Ser Leu Arg
65                  70                  75                  80

GGA GTG ATC AAG GTA TGT GGT CAC ACT TGA ATC ACG CAG GTG TGT GAA          293
Gly Val Ile Lys Val Cys Gly His Thr Xaa Ile Thr Gln Val Cys Glu
                85                  90                  95

ATC TCA TTG TGA ACA AAT AAA AAT CAT GAA AGG AAA ACT CTA TGT TTG          341
Ile Ser Cys Xaa Thr Asn Lys Asn His Glu Arg Lys Thr Leu Cys Leu
            100                 105                 110

AAA TAT CTT ATG GGT CCT CCT GTA AAG CTC TTC ACT CCA TAA GGT GAA          389
Lys Tyr Leu Met Gly Pro Pro Val Lys Leu Phe Thr Pro Xaa Gly Glu
        115                 120                 125

ATA GAC CTG AAA TAT ATA TAG ATT ATT T                                    417
Ile Asp Leu Lys Tyr Ile Xaa Ile Ile
    130                 135
```

INTERNATIONAL SEARCH REPORT

Inte...tional application No.
PCT/US95/14974

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 48/00, 38/18
US CL :424/93.21; 514/12, 44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.21; 514/12, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FASEB Journal, Volume 8, Numbers 4-5, issued 1994, Danilenko et al, "Neu Differentiation Factor (NDEF) Accelerates Epidermal Migration and Differentiation in Excisional Wounds", page A535, see entire abstract.	36-37
A	BioEssays, Volume 15, Number 12, issued December 1993, Peles et al, "Neu and its Ligands: From an Oncogene to Neural Factors", pages 815-824, see entire document.	1-37
A, P	US, A, 5,367,060 (VANDLEN ET AL) 22 November 1994, see column 37, line 54 to column 39, line 45.	1-2, 7-32, 35-37

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 FEBRUARY 1996	14 MAR 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KAREN E. BROWN Telephone No. (703) 308-0196

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SDOCID: <WO___9615812A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14974

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. P. BROCKES et al, "The Neuron as a Source of Mitogen: Its Influence on the Proliferation of Glial and Non-neural Cells," from "Development in the Nervous System", published 1980, Garrod and Feldman, (CA), pages 309-327, see entire document.	1-2, 7-32, 35-37
A, P	US, A, 5,399,346 (ANDERSON ET AL) 21 March 1995, see entire document.	3-6, 33-34
A	Proc. Natl. Acad. Sci. USA, Volume 89, issued April 1992, Yao et al, "Expression of Human Factor IX in Mice after Injection of Genetically Modified Myoblasts", pages 3357-3361, see entire document.	3-6, 33-34
A, P	SPECIAL NEWS REPORT, Volume 269, issued 25 August 1995, Marshall, "Gene Therapy's Growing Pains", pages 1050-1055, see entire document.	3-6, 33-34
A	US, A, 5,082,670 (GAGE ET AL) issued 21 January 1992, see entire document.	3-6, 33-34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14974

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS/USPAT, MEDLINE, BIOSIS, WORLD PATENT INDEX
Search terms: neuregulin#, heregulin#, glial growth factor#, acetylcholine receptor inducing activity, ARIA, neu differentiation factor#

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s)1-2, 7-32, and 35-37, drawn to a method of affecting cellular communication in a vertebrate by administering a neuregulin protein.

Group II, claim(s) 3-6 and 33-34, drawn to a method of affecting cellular communication in a vertebrate by adminstering neuregulin-producing cells or neuregulin DNA (gene therapy).

The inventions listed as Groups I-II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of Group I is the administration of neuregulin protein to vertebrates. The special technical feature of Group II is the administration of neuregulin DNA either directly (in vivo gene therapy) or after introduction of DNA into a cell (ex vivo gene therapy). The method of Group II does not require the administration of neuregulin protein and the method of Group I does not require the administration of neuregulin DNA. Furthermore, the method steps for administration of protein and DNA are different. Therefore, both the reagents used and the process steps of Groups I and II are distinct from one another and the special technical features of each method distinguish the two groups from one another.Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule13.2 so as to form a single inventive concept.